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CERTIFICATE OF ANALYSIS

EP1702 DreamTaq Hot Start DNA Polymerase, 500 U

Packaging Lot: **2736997**
Expiry Date: 30.04.2025 (DD.MM.YYYY)
Storage: at -20±5°C

Filling lots for components in package:

Lot	Quantity	Description
2712757	0.5 kU	DreamTaq HS DNA Polymerase
2686102	2 x 1.25 mL	10X DreamTaq Buffer

QUALITY CONTROL

Parameter	Method	Requirement	Result
Unit concentration	One unit of enzyme incorporates 10 nmoles of dNTPs into a polynucleotide fraction at 74 ºC in 30 minutes.	5.5 ± 0.5 U/µl	Conforms
Endodeoxyribonuclease Assay	No detectable conversion of supercoiled plasmid DNA to a nicked form was observed.	Not detectable	Conforms
Residual Activity Assay	No detectable extension of labeled double stranded oligonucleotide with 5'-overhangs after incubation in the presence of dNTPs.	Not detectable	Conforms
E. coli DNA	No detectable E.coli DNA was observed.	Not detectable	Conforms
Functional Assay	Performance in PCR is tested by the amplification of a 594 bp and 7.5 kb fragments of human genomic DNA.	Reactions produce specific PCR products	Conforms

ISO CERTIFICATION
Manufactured by Thermo Fisher Scientific Baltics UAB, in compliance with ISO 9001 and ISO 13485 certified quality management system.

Quality authorized by QC: J. Žilinskienė
Digitally signed by Ceaicovschi Tudor
Date: 2023.06.30 14:06:10 EEST
Reason: MoldSign Signature
Location: Moldova

Thermo SCIENTIFIC

PRODUCT INFORMATION

Thermo Scientific DreamTaq Hot Start DNA Polymerase

Pub. No. MAN0015972
Rev. Date 29 July 2016 (Rev. A.00)

Lot: __ Expiry Date: __

Ordering Information

Catalog No.	DreamTaq Hot Start DNA Polymerase, 5 U/µL	10X DreamTaq Buffer*
EP1701	200 U	1.25 mL
EP1702	500 U	2 × 1.25 mL
EP1703	2500 U	10 × 1.25 mL
EP1704	4 × 2500 U	40 × 1.25 mL

* includes 20 mM $MgCl_2$

Store at –20°C

www.thermofisher.com

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

DESCRIPTION

Thermo Scientific™ DreamTaq™ Hot Start DNA Polymerase is an enhanced hot start *Taq* DNA polymerase optimized for most PCR applications. It ensures higher sensitivity, specificity, and yields compared to conventional hot start *Taq* DNA polymerase. It is capable of amplifying long amplicons such as 6 kb genomic DNA and 20 kb λ DNA.

DreamTaq Hot Start DNA Polymerase combines *Taq* DNA polymerase and a specific antibody that inhibits the DNA polymerase activity at ambient temperatures, thus preventing the amplification of non-specific products. At polymerization temperatures, the antibody molecule is released, rendering the polymerase fully active.

DreamTaq Hot Start DNA Polymerase uses the same reaction set-up and cycling conditions as conventional *Taq* DNA polymerases, but the antibody-based hot start allows the reactions to be set up at room temperature. Because the enzyme is supplied with the optimized DreamTaq buffer, which includes 20 mM $MgCl_2$, extensive optimization of reaction conditions is not required.

DreamTaq Hot Start DNA Polymerase generates PCR products with 3'-dA overhangs. The enzyme tolerates dUTP and can incorporate modified nucleotides.

FEATURES

- High specificity due to antibody based hot start.
- Robust amplification with minimal optimization.
- High yields of PCR products.
- Higher sensitivity compared to conventional hot start *Taq* DNA polymerases.
- Amplification of long targets up to 6 kb from genomic DNA and up to 20 kb from viral DNA.
- Generates 3'-dA overhangs.
- Incorporates dUTP and modified nucleotides.

APPLICATIONS

- Routine PCR amplification of DNA fragments up to 6 kb from genomic DNA and up to 20 kb from viral DNA.
- RT-PCR.
- Genotyping.
- Generation of PCR products for TA cloning.

CONCENTRATION

5 U/µL

DEFINITION OF ACTIVITY UNIT

One unit of the enzyme catalyzes the incorporation of 10 nmol of deoxyribonucleotides into a polynucleotide fraction in 30 minutes at 74°C.

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10X DREAMTAQ BUFFER

DreamTaq Buffer is a proprietary formulation, which contains KCl and $(NH_4)_2SO_4$ at a ratio optimized for robust performance of DreamTaq Hot Start DNA Polymerase in PCR. DreamTaq Buffer also includes $MgCl_2$ at a concentration of 20 mM.

INHIBITION AND INACTIVATION

- Inhibitors: Ionic detergents (deoxycholate, sarkosyl, and SDS) at concentrations higher than 0.06, 0.02, and 0.01%, respectively.
- Inactivated by phenol/chloroform extraction.

PROTOCOL

To set up parallel reactions and to minimize the possibility of pipetting errors, prepare a PCR master mix by mixing water, buffer, dNTPs, primers, and DreamTaq Hot Start DNA Polymerase. Prepare sufficient master mix for the number of reactions plus one extra. Aliquot the master mix into individual PCR tubes, then add template DNA.

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. For each 50-µL reaction, add the following components into a thin-walled PCR tube:

10X DreamTaq Buffer*	5 µL
dNTP Mix, 2 mM each (#R0241)	5 µL (0.2 mM of each)
Forward primer	0.1–1.0 µM
Reverse primer	0.1–1.0 µM
Template DNA	10 pg–1 µg
DreamTaq Hot Start DNA Polymerase	1.25 U
Water, nuclease-free (#R0581)	to 50 µL
Total volume	50 µL

*10X DreamTaq Buffer contains 20 mM $MgCl_2$, which is optimal for most applications. If further optimization is required, additional $MgCl_2$ can be added to the master mix. The volume of water should be reduced accordingly.

Volumes of 25 mM $MgCl_2$ (#R0971), required for specific final $MgCl_2$ concentration:

Final concentration of $MgCl_2$	2 mM	2.5 mM	3 mM	4 mM
Volume of 25 mM $MgCl_2$ to be added for 50-µL reaction	0 µL	1 µL	2 µL	4 µL

3. Gently vortex the samples and briefly centrifuge.
4. When using a thermal cycler that does not contain a heated lid, overlay the reaction mixture with 25 µL of mineral oil.
5. Place the reactions in a thermal cycler. Perform PCR using the recommended thermal cycling conditions outlined below:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	1–3 min	1
Denaturation	95	30 s	25–40
Annealing	Tm	30 s	
Extension*	72	1 min	
Final Extension	72	5–15 min	1

* The recommended extension step is 1 minute for PCR products up to 2 kb. For longer products, the extension time should be prolonged by 1 minute/kb.

GUIDELINES FOR PREVENTING CONTAMINATION OF PCR REACTION

During PCR more than 10 million copies of template DNA are generated. Therefore, care must be taken to avoid contamination with other templates and amplicons that may be present in the laboratory environment. Follow the general recommendations below to lower the risk of contamination.

- Prepare your DNA sample, set up the PCR mixture, perform thermal cycling and analyze PCR products in separate areas.
- Set up PCR mixtures in a laminar flow cabinet equipped with an UV lamp.
- Wear fresh gloves for DNA purification and reaction set up.
- Use reagent containers dedicated for PCR. Use positive displacement pipettes, or use pipette tips with aerosol filters to prepare DNA samples and perform PCR set up.
- Use PCR-certified reagents, including high quality water (e.g., Water, nuclease-free, #R0581).
- Always perform "no template control" (NTC) reactions to check for contamination.

DreamTaq Hot Start DNA Polymerase incorporates dUTP; therefore, you can control carry-over contamination using Uracil-DNA Glycosylase (#EN0361).

GUIDELINES FOR PRIMER DESIGN

Use special design software or follow the general recommendations for PCR primer design as outlined below to design optimal primers:

- Use PCR primers that are 15–30 nucleotides long.
- Optimal GC content of the primer is 40 –60%. Ideally, C and G nucleotides should be distributed uniformly along the primer.
- Avoid placing more than three G or C nucleotides at the 3'-end to lower the risk of non-specific priming.
- If possible, the primer should terminate with a G or C at the 3'-end.

(continued on reverse page)

- Avoid self-complementary primer regions, and complementarities between the primers and direct primer repeats to prevent hairpin formation and primer dimerization.
- Check for possible sites of undesired complementarity between primers and template DNA.
- When designing degenerate primers, place at least 3 conserved nucleotides at the 3'-end.
- Differences in melting temperatures (Tm) between the two primers should not exceed 5°C.

ESTIMATION OF PRIMER MELTING TEMPERATURE

For primers containing less than 25 nucleotides, the approximate melting temperature (Tm) can be calculated using the following equation:

$$Tm = 4 (G + C) + 2 (A + T),$$

where G, C, A, T represent the number of respective nucleotides in the primer.

If the primer contains more than 25 nucleotides, we recommend using specialized computer programs to account for interactions of adjacent bases, effect of salt concentration, etc.

COMPONENTS OF THE REACTION MIXTURE

Template DNA

Optimal amount of template DNA for a 50-µL reaction volume is 1 pg–1 ng for both plasmid and phage DNA, and 100 pg–1 µg for genomic DNA. Higher amounts of template increase the risk of non-specific PCR products. Lower amounts of template reduce the accuracy of the amplification.

All routine DNA purification methods are suitable for template preparation; e.g., Thermo Scientific™ GeneJET™ Genomic DNA Purification Kit (#K0721) or GeneJET Plasmid Miniprep Kit (#K0502). Trace amounts of certain agents used for DNA purification, such as phenol, EDTA, and proteinase K, can inhibit DNA polymerases. Ethanol precipitation and repeated washes of the DNA pellet with 70% ethanol normally removes trace contaminants from DNA samples.

$MgCl_2$ concentration

DreamTaq Hot Start DNA Polymerase is provided with an optimized 10X DreamTaq Buffer, which includes $MgCl_2$ at a concentration of 20 mM. A final $MgCl_2$ concentration of 2 mM is generally ideal for PCR. $MgCl_2$ concentration can be further increased up to 4 mM by the addition of 25 mM $MgCl_2$ (#R0971).

If the DNA samples contain EDTA or other metal chelators, Mg^{2+} ion concentration in the PCR mixture should be increased accordingly (1 molecule of EDTA binds 1 Mg^{2+}).

dNTPs

The recommended final concentration of each dNTP is 0.2 mM. In certain PCR applications, higher dNTP concentrations may be necessary. It is essential to have equal concentrations of all four nucleotides (dATP, dCTP, dGTP and dTTP) in the reaction mixture.

To obtain a 0.2 mM concentration of each dNTP in the PCR mixture, refer to the table below.

Volume of PCR mixture	dNTP Mix, 2 mM each (#R0241)	dNTP Mix, 10 mM each (#R0191)	dNTP Mix, 25 mM each (#R1121)
50 µL	5 µL	1 µL	0.4 µL
25 µL	2.5 µL	0.5 µL	0.2 µL
20 µL	2 µL	0.4 µL	0.16 µL

Use 200 µM of each dNTP. dUTP or dITP can be added up to 200 µM. For longer amplicons, a lower dUTP concentration (20–100 µM) may be required for high yields.

Primers

The recommended concentration range of the PCR primers is 0.1–1 µM. Excessive primer concentrations increase the probability of mispriming and generation of non-specific PCR products.

For degenerate primers and primers used for long PCR, we recommend higher primer concentrations in the range of 0.3–1 µM.

CYCLING PARAMETERS

Initial DNA denaturation and enzyme activation

DreamTaq Hot Start DNA polymerase is inactive at room temperature during the reaction set up and is activated during the 1–3 minute initial denaturation/enzyme activation step.

It is essential to completely denature the template DNA at the beginning of the PCR run to ensure efficient utilization of the template during the first amplification cycle. If the GC content of the template is 60% or less, an initial 1–3 minute denaturation at 95°C is sufficient. For GC-rich templates this step can be prolonged.

Denaturation

A DNA denaturation time of 30 seconds per cycle at 95°C is normally sufficient. For GC-rich DNA templates, this step can be prolonged to 3–4 minutes. DNA denaturation can also be enhanced by the addition of 5–10% glycerol, 5% DMSO, 1% formamide, or 1–1.5 M betaine. The melting temperature of the primer-template complex decreases significantly in the presence of these reagents. Therefore, the annealing temperature has to be adjusted accordingly.

Note that higher than 10% DMSO or 5% formamide in the reaction mix inhibit DNA polymerases. Therefore, it may be necessary to increase the amount of the enzyme in the reaction if these additives are used.

Primer annealing

The annealing temperature should be equal to the melting temperature (Tm) of the primers. Annealing for 30 seconds is normally sufficient. If non-specific PCR products appear, the annealing temperature should be optimized stepwise in 1-2°C increments. When additives that change the melting temperature of the primer-template complex are used (glycerol, DMSO, formamide and betaine), the annealing temperature must also be adjusted.

Extension

The optimal extension temperature for DreamTaq Hot Start DNA Polymerase is 70–75°C. The recommended extension step is 1 minute at 72°C for PCR products up to 2 kb. For longer products, the extension time should be increased by 1 minute/kb. For amplification of templates >6 kb, we recommend reducing the extension temperature to 68°C.

Number of cycles

The number of cycles may vary depending on the amount of template DNA in the PCR mixture and the expected PCR product yield.

If less than 10 copies of the template is present in the reaction, about 40 cycles are required. For higher template amounts, 25–35 cycles are sufficient.

Final extension

After the last cycle, we recommend incubating the PCR mixture at 72°C for an additional 5–15 minutes to fill in any possible incomplete reaction products. If the PCR product will be cloned into TA vectors such as the Thermo Scientific™ InsTAclone™ PCR Cloning Kit (#K1213), the final extension step may be prolonged to 15 minutes to ensure the complete 3'-dA tailing of the PCR product. If the PCR product will be used for cloning using Thermo Scientific™ CloneJET™ PCR Cloning Kit (#K1231), the final extension step can be omitted.

TROUBLESHOOTING

For troubleshooting, visit **www.thermofisher.com.**

CERTIFICATE OF ANALYSIS

Endodeoxyribonuclease Assay

No detectable conversion of supercoiled plasmid DNA to a nicked form was observed.

Residual Activity Assay

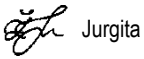
No detectable extension of labeled double stranded oligonucleotide with 5'-overhangs after incubation in the presence of dNTPs.

E. coli DNA Assay

No detectable E.coli DNA was observed.

Functional Assay

Performance in PCR is tested by the amplification of a 594 bp and 7.5 kb fragments of human genomic DNA.

Quality authorized by:  Jurgita Zilinskiene

LIMITED USE LABEL LICENSE No. 593: Newcastle License for Modified DNA Polymerase

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PRODUCT USE LIMITATION

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Refer to **www.thermofisher.com/support** for the Safety Data Sheets.

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CERTIFICATE OF ANALYSIS

ER0542 MspI (HpaII)
Packaging Lot: 2744434
Expiry Date: 31.05.2027 (DD.MM.YYYY)
Storage: at -20±5°C

Filling lots for components in package:

Lot	Quantity	Description
2734380	5 x 3 kU	MspI (HpaII)
2715277	2 x 1 mL	10x Buffer Tango

QUALITY CONTROL

Parameter	Method	Requirement	Result
Concentration	One unit is defined as the amount of enzyme required to digest 1 µg of lambda DNA in 1 hour at 37 °C in 50 µL of assay buffer.	10 - 12 U/µL	Conforms
Endo-exodeoxyribonucleases and phosphatases	Incubation of single stranded and double stranded labeled oligonucleotides with appropriate amount of enzyme for 4 hour at 37 °C in assay buffer.	Not detectable	Conforms
Star Activity	After 160-fold overdigestion (10 U/µg lambda DNA for 16 hours at 37 °C) with enzyme the fragmentation pattern is analysed. No detectable changes compared to the theoretical fragmentation pattern are considered as absence of star activity.	Not detectable	Conforms

ISO CERTIFICATION
Manufactured by Thermo Fisher Scientific Baltics UAB, in compliance with ISO 9001 and ISO 13485 certified quality management system.

Quality authorized by QC: J. Žilinskienė

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PRODUCT INFORMATION

MspI (HpaII)

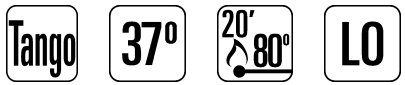
#ER0541 3000 U

Lot: ___ **Expiry Date: _**

5'...**C↓C G G**...3'
3'...**G G C↑C**...5'

Concentration: 10 U/µL
Source: *Moraxella species*
Supplied with: 2x1 mL of 10X Buffer Tango

Store at -20°C



In total 3 vials. BSA included

www.thermoscientific.com/onebio

RECOMMENDATIONS

1X Thermo Scientific Tango Buffer (for 100% MspI digestion)

33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA.

Incubation temperature

37°C.

Unit Definition

One unit is defined as the amount of MspI required to digest 1 µg lambda DNA in 1 hour at 37°C in 50 µL of recommended reaction buffer.

Dilution

Dilute with the Dilution Buffer (#B19): 10 mM Tris-HCl (pH 7.4 at 25°C), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2 mg/mL BSA and 50% glycerol.

Double Digests

Tango™ Buffer provided simplifies buffer selection for double digests. 98% of Thermo Scientific restriction enzymes are active in a 1X or 2X concentration of Tango Buffer. Please go to www.thermoscientific.com/doubledigest to choose the best buffer for your experiments.

Storage Buffer

MspI is supplied in: 10 mM potassium phosphate (pH 7.5 at 25°C), 200 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.2 mg/mL BSA and 50% glycerol.

Rev.10

Recommended Protocol for Digestion

- Add:

nuclease-free water	16 µL
10X Buffer Tango	2 µL
DNA (0.5-1 µg/µL)	1 µL
MspI	0.5-2 µL

- Mix gently and spin down for a few seconds.
- Incubate at 37°C for 1-16 hours.

The digestion reaction may be scaled either up or down.

Recommended Protocol for Digestion of PCR Products Directly after Amplification

- Add:

PCR reaction mixture	10 µL	(~0.1-0.5 µg of DNA)
nuclease-free water	18 µL	
10X Buffer Tango	2 µL	
MspI	1-2 µL	

- Mix gently and spin down for a few seconds.
- Incubate at 37°C for 1-16 hours.

Thermal Inactivation

MspI is inactivated by incubation at 80°C for 20 min.

ENZYME PROPERTIES

Enzyme Activity in Thermo Scientific REase Buffers, %

B	G	O	R	Tango	2X Tango
50-100	50-100	0-20	0-20	**100**	50-100

Methylation Effects on Digestion

Dam: never overlaps – no effect.
Dcm: never overlaps – no effect.
CpG: completely overlaps – no effect.
EcoKI: never overlaps – no effect.
EcoBI: never overlaps – no effect.

Stability during Prolonged Incubation

A minimum of 0.3 units of the enzyme is required for complete digestion of 1 µg of lambda DNA in 16 hours at 37°C.

Compatible Ends

Bsp119I, Bsu15I, Hin1I, Hin6I, HpaII, MaeII, NarI, Psp1406I, SsiI, TaqI, XmiI.

Number of Recognition Sites in DNA

λ	ΦX174	pBR322	pUC57	pUC18/19	pTZ19R/U	M13mp18/19
328	5	26	13	13	12	18

Note

MspI is an isoshizomer of HpaII. When the external C in the sequence CCGG is methylated, MspI and HpaII cannot cleave. However, unlike HpaII, MspI can cleave the sequence when the internal C residue is methylated.

For **CERTIFICATE OF ANALYSIS** *see* back page

CERTIFICATE OF ANALYSIS

Overdigestion Assay

No detectable change in the specific fragmentation pattern is observed after a 160-fold overdigestion with MspI (10 U/µg lambda DNA × 16 hours).

Ligation and Recleavage (L/R) Assay

The ligation and recleavage assay was replaced with LO test after validating experiments showed LO test ability to trace nuclease and phosphatase activities with sensitivity that is higher than L/R by a factor of 100.

Labeled Oligonucleotide (LO) Assay

No detectable degradation of single-stranded or double-stranded labeled oligonucleotides occurred during incubation with 10 units of MspI for 4 hours.

Quality authorized by:  Jurgita Zilinskiene

PRODUCT USE LIMITATION

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

Please refer to www.thermoscientific.com/onebio for Material Safety Data Sheet of the product.

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CERTIFICATE OF ANALYSIS

ER1621 **LweI (SfaNI)**
Packaging Lot: **2737061**
Expiry Date: 31.10.2025 (DD.MM.YYYY)
Storage: at -20±5°C

Filling lots for components in package:

Lot	Quantity	Description
2737061	0.1 kU	LweI (SfaNI)
2715277	1 mL	10x Buffer Tango

QUALITY CONTROL

Parameter	Method	Requirement	Result
Concentration	One unit is defined as the amount of enzyme required to digest 1 µg of lambda DNA in 1 hour at 37 °C in 50 µL of the assay buffer.	10 - 12 U/µL	Conforms
Endo-exodeoxyribonucleases and phosphatases	Incubation of single stranded and double stranded labeled oligonucleotides with appropriate amount of enzyme for 4 hour at 37 °C in assay buffer.	Not detectable	Conforms
Star Activity	After 160-fold overdigestion (10 U/µg lambda DNA for 16 hours at 37 °C in assay buffer) with enzyme the fragmentation pattern is analysed. No detectable changes compared to the theoretical fragmentation pattern are considered as absence of star activity.	Not detectable	Conforms

ISO CERTIFICATION
Manufactured by Thermo Fisher Scientific Baltics UAB, in compliance with ISO 9001 and ISO 13485 certified quality management system.

Quality authorized by QC: J. Žilinskienė

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PRODUCT INFORMATION

LweI (SfaNI)

#ER1622 500 U

Lot: ___ **Expiry Date: _**

5'...**G C A T C (N)$_5$**↓...3'
3'...**C G T A G (N)$_9$**↑...5'

Concentration: 10 U/µL
Source: *E.coli* that carries the cloned *lweIR* gene from *Listeria welshimeri* RFL131
Supplied with: 1 mL of 10X Buffer Tango

Store at -20°C

BSA included

www.thermoscientific.com/onebio

RECOMMENDATIONS

1X Thermo Scientific Tango Buffer (for 100% LweI digestion)
33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA.

Incubation temperature
37°C.

Unit Definition
One unit is defined as the amount of LweI required to digest 1 µg of lambda DNA in 1 hour at 37°C in 50 µL of recommended reaction buffer.

Dilution
Dilute with Dilution Buffer (#B19): 10 mM Tris-HCl (pH 7.4 at 25°C), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2 mg/mL BSA and 50% glycerol.

Double Digests
Tango™ Buffer provided simplifies buffer selection for double digests. 98% of Thermo Scientific restriction enzymes are active in a 1X or 2X concentration of Tango Buffer. Please go to www.thermoscientific.com/doubledigest to choose the best buffer for your experiments.

Storage Buffer
LweI is supplied in: 10 mM Tris-HCl (pH 7.5 at 25°C), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.2 mg/mL BSA and 50% glycerol.

Recommended Protocol for Digestion

- Add:

nuclease-free water	16 µL
10X Buffer Tango	2 µL
DNA (0.5-1 µg/µL)	1 µL
LweI	0.5-2 µL

- Mix gently and spin down for a few seconds.
- Incubate at 37°C for 1-2 hours.

The digestion reaction may be scaled either up or down.

Recommended Protocol for Digestion of PCR Products Directly after Amplification

- Add:

PCR reaction mixture	10 µL	(~0.1-0.5 µg of DNA)
nuclease-free water	18 µL	
10X Buffer Tango	2 µL	
LweI	1-2 µL	

- Mix gently and spin down for a few seconds.
- Incubate at 37°C for 1-16 hours.

Thermal Inactivation

LweI is inactivated by incubation at 65°C for 20 min.

ENZYME PROPERTIES

Enzyme Activity in Thermo Scientific REase Buffers, %

B	G	O	R	Tango	2X Tango
0-20	0-20	0-20	20-50	**100**	20-50

Methylation Effects on Digestion

Dam: never overlaps – no effect.
Dcm: never overlaps – no effect.
CpG: may overlap – cleavage impaired.
EcoKI: never overlaps – no effect.
EcoBI: may overlap – effect not determined.

Stability during Prolonged Incubation

A minimum of 0.2 units of the enzyme is required for complete digestion of 1 µg of lambda DNA in 16 hours at 37°C.

Number of Recognition Sites in DNA

λ	ΦX174	pBR322	pUC57	pUC18/19	pTZ19R/U	M13mp18/19
169	12	22	9	8	4	7

Note

- At least two copies of LweI recognition site are required for efficient cleavage.
- LweI may remain associated with the cleaved DNA. This may cause DNA band shifting during electrophoresis. To avoid atypical DNA band patterns, use the 6X DNA Loading Dye&SDS Solution (#R1151) for sample preparation or heat the digested DNA in the presence of SDS prior to electrophoresis.

For **CERTIFICATE OF ANALYSIS** *see* back page

CERTIFICATE OF ANALYSIS

Overdigestion Assay

No detectable change in the specific fragmentation pattern is observed after a 160-fold overdigestion with LweI (10 U/µg lambda DNA x 16 hours).

Ligation and Recleavage (L/R) Assay

The ligation and recleavage assay was replaced with LO test after validating experiments showed LO test ability to trace nuclease and phosphatase activities with sensitivity that is higher than L/R by a factor of 100.

Labeled Oligonucleotide (LO) Assay

No detectable degradation of single-stranded or double-stranded labeled oligonucleotides occurred during incubation with 10 units of LweI for 4 hours.

Quality authorized by:  Jurgita Zilinskiene

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PRODUCT INFORMATION

Bsp143I (Sau3AI)

#ER0781 300 U

Lot: ___ **Expiry Date: _**

5'…↓**G A T C** …3'
3'… **C T A G**↑…5'

Concentration:	10 U/µL
Source:	*Bacillus species* RFL143
Supplied with:	1 mL of 10X Buffer Bsp143I
	1 mL of 10X Buffer Tango

Store at -20°C

BSA included

www.thermoscientific.com/onebio

RECOMMENDATIONS

1X Buffer Bsp143I (for 100% Bsp143I digestion)
33 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 66 mM potassium acetate, 0.02% Triton X-100, 0.1mg/mL BSA.

Incubation temperature
37°C.

Unit Definition
One unit is defined as the amount of Bsp143I required to digest 1 µg of lambda DNA in 1 hour at 37°C in 50 µL of recommended reaction buffer.

Dilution
Dilute with Dilution Buffer (#B19): 10 mM Tris-HCl (pH 7.4 at 25°C), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2 mg/mL BSA and 50% glycerol.

Double Digests
Thermo Scientific Tango Buffer is provided to simplify buffer selection for double digests. 98% of Thermo Scientific restriction enzymes are active in a 1X or 2X concentration of Tango™ Buffer. Please refer to www.thermoscientific.com/doubledigest to choose the best buffer for your experiments.
1X Tango Buffer: 33 mM Tris-acetate (pH 7.9 at 37°C), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA.

Storage Buffer

Bsp143I is supplied in: 10 mM Tris-HCl (pH 7.4 at 25°C), 100 mM KCl, 1 mM DTT, 1 mM EDTA, 0.2 mg/mL BSA and 50% glycerol.

Recommended Protocol for Digestion

- Add:

nuclease-free water	16 µL
10X Buffer Bsp143I	2 µL
DNA (0.5-1 µg/µL)	1 µL
Bsp143I	0.5-2 µL

- Mix gently and spin down for a few seconds.
- Incubate at 37°C for 1-16 hours.

The digestion reaction may be scaled either up or down.

Recommended Protocol for Digestion of PCR Products Directly after Amplification

- Add:

PCR reaction mixture	10 µL (~0.1-0.5 µg of DNA)
nuclease-free water	18 µL
10X Buffer Bsp143I	2 µL
Bsp143I	1-2 µL

- Mix gently and spin down for a few seconds.
- Incubate at 37°C for 1-16 hours.

Thermal Inactivation

Bsp143I is inactivated by incubation at 65°C for 20 min.

ENZYME PROPERTIES

Enzyme Activity in Thermo Scientific REase Buffers, %

Bsp143I	B	G	O	R	Tango	2X Tango
100	20-50	20-50	0-20	0-20	50-100	20-50

Methylation Effects

Dam: completely overlaps – no effect.
Dcm: never overlaps – no effect.
CpG: may overlap – blocked.
EcoKI: never overlaps – no effect.
EcoBI: may overlap – no effect.

Stability during Prolonged Incubation

A minimum of of 0.1 units of the enzyme is required for complete digestion of 1 µg of lambda DNA in 16 hours at 37°C.

Compatible Ends

BamHI, BclI, BglII, PsuI.

Number of Recognition Sites in DNA

λ	ΦX174	pBR322	pUC57	pUC18/19	pTZ19R/U	M13mp18/19
116	0	22	15	15	15	7

Note

DpnI, Bsp143I and MboI all recognize the same sequence but have different methylation sensitivities and cleavage sites.

For **CERTIFICATE OF ANALYSIS** *see* back page

CERTIFICATE OF ANALYSIS

Overdigestion Assay

No detectable change in the specific fragmentation pattern is observed after a 160-fold overdigestion with Bsp143I (10 U/µg lambda DNA x 16 hours).

Ligation and Recleavage (L/R) Assay

The ligation and recleavage assay was replaced with LO test after validating experiments showed LO test ability to trace nuclease and phosphatase activities with sensitivity that is higher than L/R by a factor of 100.

Labeled Oligonucleotide (LO) Assay

No detectable degradation of single-stranded and double-stranded labeled oligonucleotides occurred during incubation with 10 units of Bsp143I for 4 hours.

Quality authorized by: Jurgita Zilinskiene

PRODUCT USE LIMITATION

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

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Company code 122351387, VAT code LT223513811

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CERTIFICATE OF ANALYSIS

ER0781 **Bsp143I (Sau3AI)**
Packaging Lot: **2750056**
Expiry Date: 31.10.2025 (DD.MM.YYYY)
Storage: at -20±5°C

Filling lots for components in package:

Lot	Quantity	Description
2750056	0.3 kU	Bsp143I (Sau3AI)
2746044	1 mL	10X Buffer Bsp143I
2715277	1 mL	10x Buffer Tango

QUALITY CONTROL

Parameter	Method	Requirement	Result
Concentration	One unit is defined as the amount of enzyme required to digest 1 µg of lambda DNA fragments in 1 hour at 37 °C in 50 µl of assay buffer.	10 - 12 u/µl	Conforms
Endo-, exodeoxyribonucleases and phosphatases	Incubation of single stranded and double stranded labeled oligonucleotides with appropriate amount of enzyme for 4 hour at 37 °C in assay buffer.	Not detectable	Conforms
Star Activity	After 160-fold overdigestion (10 u/µg lambda DNA, φX174 DNA for 16 hours at 37 °C in assay buffer) with enzyme the fragmentation pattern is analysed. No detectable changes compared to the theoretical fragmentation pattern are considered as absence of star activity.	Not detectable	Conforms

ISO CERTIFICATION
Manufactured by Thermo Fisher Scientific Baltics UAB, in compliance with ISO 9001 and ISO 13485 certified quality management system.

Quality authorized by QC: J. Žilinskienė

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CERTIFICATE OF ANALYSIS

ER1102 HphI
Packaging Lot: 2744525
Expiry Date: 28.02.2027 (DD.MM.YYYY)
Storage: at -20±5°C

Filling lots for components in package:

Lot	Quantity	Description
2744525	1.5 kU	HphI
2723811	1 mL	10x Buffer B
2715277	1 mL	10x Buffer Tango

QUALITY CONTROL

Parameter	Method	Requirement	Result
Concentration	One unit is defined as the amount of enzyme required to digest 1 µg of lambda DNA (dam-, dcm-) in 1 hour at 37 °C in 50 µL of assay buffer.	10 - 12 U/µL	Conforms
Star Activity	After 80-fold overdigestion (5 U/µg lambda DNA (dam-, dcm-) for 16 hours at 37 °C) with enzyme the fragmentation pattern is analysed. No detectable changes compared to the theoretical fragmentation pattern are considered as absence of star activity.	Not detectable	Conforms

ISO CERTIFICATION
Manufactured by Thermo Fisher Scientific Baltics UAB, in compliance with ISO 9001 and ISO 13485 certified quality management system.

Quality authorized by QC: J. Žilinskienė

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PRODUCT INFORMATION

HphI

#ER1101 300 U

Lot: ___ **Expiry Date: _**

5'...**G G T G A (N)$_8$**↓...3'
3'...**C C A C T (N)$_7$**↑...5'

Concentration: 10 U/µL
Source: *E.coli* that carries the cloned *hphIR* gene from *Haemophilus parahaemolyticus*
Supplied with: 1 mL of 10X Buffer B
1 mL of 10X Buffer Tango

Store at -20°C

BSA included

www.thermoscientific.com/onebio

RECOMMENDATIONS

1X Buffer B (for 100% HphI digestion)
10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mg/mL BSA.

Incubation temperature
37°C.

Unit Definition
One unit is defined as the amount of HphI required to digest 1 µg of lambda DNA *dam*$^-$ in 1 hour at 37°C in 50 µL of recommended reaction buffer.

Dilution
Dilute with Dilution Buffer (#B19): 10 mM Tris-HCl (pH7.4 at 25°C), 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.2 mg/mL BSA and 50% glycerol.

Double Digests
Thermo Scientific Tango Buffer is provided to simplify buffer selection for double digests. 98% of Thermo Scientific restriction enzymes are active in a 1X or 2X concentration of Tango™ Buffer. Please refer to www.thermoscientific.com/doubledigest to choose the best buffer for your experiments.
1X Tango Buffer: 33 mM Tris-acetate (pH 7.9 at 37°C), 10 mM magnesium acetate, 66 mM potassium acetate, 0.1 mg/mL BSA.

Storage Buffer
HphI is supplied in: 10 mM Tris-HCl (pH 7.5 at 25°C), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.2 mg/mL BSA and 50% glycerol.

Recommended Protocol for Digestion

- Add:

nuclease-free water	16 µL
10X Buffer B	2 µL
DNA (0.5-1 µg/µL)	1 µL
HphI	0.5-2 µL*

- Mix gently and spin down for a few seconds.
- Incubate at 37°C for 1-16 hours*.

The digestion reaction may be scaled either up or down.

Recommended Protocol for Digestion of PCR Products Directly after Amplification

- Add:

PCR reaction mixture	10 µL (~0.1-0.5 µg of DNA)
nuclease-free water	18 µL
10X Buffer B	2 µL
HphI	1-2 µL*

- Mix gently and spin down for a few seconds.
- Incubate at 37°C for 1-16 hours*.

---

* *See* Overdigestion Assay.

Thermal Inactivation

HphI is inactivated by incubation at 65°C for 20 min.

ENZYME PROPERTIES

Enzyme Activity in Thermo Scientific REase Buffers, %

B	G	O	R	Tango	2X Tango
100	0-20	0-20	0-20	20-50	0-20

Methylation Effects on Digestion

Dam: may overlap – blocked.
Dcm: may overlap – no effect.
CpG: may overlap – no effect.
EcoKI: never overlaps – no effect.
EcoBI: may overlap – blocked.

Stability during Prolonged Incubation

A minimum of 0.1 units of the enzyme is required for complete digestion of 1 µg of lambda DNA in 16 hours at 37°C.

Number of Recognition Sites in DNA

λ	ΦX174	pBR322	pUC57	pUC18/19	pTZ19R/U	M13mp18/19
168	9	12	7	7	6	18

Note

HphI is blocked by overlapping *dam* methylation. To avoid *dam* methylation, use a *dam*$^-$, *dcm*$^-$ strain such as GM2163 (#M0099).

For **CERTIFICATE OF ANALYSIS** *see* back page

CERTIFICATE OF ANALYSIS

Overdigestion Assay

No detectable change in the specific fragmentation pattern is observed after a 80-fold overdigestion with HphI (5 U/µg lambda DNA *dam*$^{-}$ x 16 hours).

Ligation and Recleavage (L/R) Assay

The ligation and recleavage assay was replaced with LO test after validating experiments showed LO test ability to trace nuclease and phosphatase activities with sensitivity that is higher than L/R by a factor of 100.

Quality authorized by:  Jurgita Zilinskiene

PRODUCT USE LIMITATION

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

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TaqMan multiplex real-time PCR

Get more data out of your sample

- A complete multiplex real-time PCR (qPCR) solution for gene expression and genotyping applications
- Applied Biosystems™ ABY™ and JUN™ dyes, QSY™ quencher, and a multiplex master mix for optimal amplification performance
- Up to 4-plex reactions—as sensitive as singleplex reactions, decreases the starting material required, and minimizes optimization processes

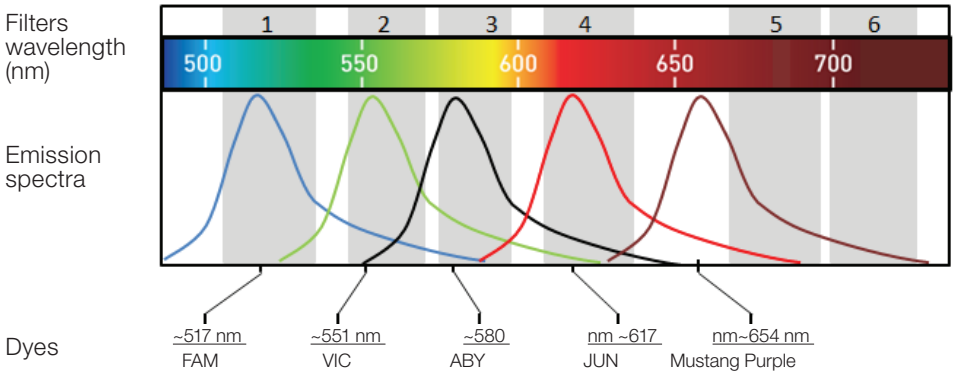

Figure 1. Fluorescence emission spectra of FAM, VIC, ABY, and JUN dyes used for multiplex real-time PCR. Grey zones represent the filters available on Applied Biosystems™ real-time PCR systems: 1 through 6 for the QuantStudio™ 7 or 12K Flex Real-Time PCR Systems; 1 through 5 for the QuantStudio™ 6 Flex Real-Time PCR System, ViiA™ 7 Real-Time PCR System, and 7500 or 7500 Fast Real-Time PCR System. MP = Mustang Purple™ dye.

Obtaining the maximum amount of genetic information from an important but small amount of sample can be challenging. This is particularly true with formalin-fixed, paraffin-embedded (FFPE) samples or tumor biopsies that are used for translational research studies. Singleplex qPCR is frequently used for these clinical research samples, but this typically has a higher cost per sample than running in multiplex format. The additional time and materials required to set up multiple single-assay reactions could also significantly increase the cost of a complex project.

Multiplex qPCR, a strategy where more than one target in a sample is amplified and quantified in a single tube, can decrease the quantity of sample material and reagents required. A complete solution for multiplex qPCR is presented here, with components designed to work together for better data quality and less time for optimization. The solution consists of the following:

- Applied Biosystems™ TaqMan® probes using QSY quencher, providing maximal PCR efficiency in a multiplex format. These probes can be ordered with Applied Biosystems™ FAM™ and VIC™ dyes and also with the ABY and JUN dyes, allowing amplification of up to 4 targets in a single reaction. These reporter dyes are optimized to work together with minimal spectral overlap for improved performance (Figure 1). In addition, the QSY quencher is fully compatible with probes that have minor-groove binder (MGB) quenchers.
- The Applied Biosystems™ TaqMan® Multiplex Master Mix was developed to allow amplification of 4 targets simultaneously, without competition between targets. This master mix contains the Applied Biosystems™ Mustang Purple™ dye, a passive reference used for normalization instead of the Applied Biosystems™ ROX™ dye, allowing for measurement of JUN dye in the channel previously used to measure ROX dye.

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- Off-the-shelf, predesigned assays—an RNase P assay using an ABY-QSY probe and a GAPDH assay using a JUN-QSY probe. Both assays are available in limited and nonlimited primer concentrations.
- Calibration plates for ABY, JUN, and Mustang Purple dyes, available in 96-well, 96-well Fast, and 384-well formats.
- Additional services provided through our custom services program—save time and let our Applied Biosystems™ TaqMan® Assay experts design your multiplex assays.

This multiplex solution is compatible with the Applied Biosystems™ QuantStudio™ 6, 7, and 12K Flex Real-Time PCR Systems, as well as the Applied Biosystems™ ViiA™ 7 Real-Time PCR System and the Applied Biosystems™ 7500 and 7500 Fast Real-Time PCR Systems.

Multiplexing without compromise

The multiplex format enables cost savings and preservation of limited sample, but it's important to obtain the same sensitivity as in the singleplex format. Figure 2 demonstrates comparable results between reactions performed in individual tubes or in 4-plex reactions for a gene quantification experiment.

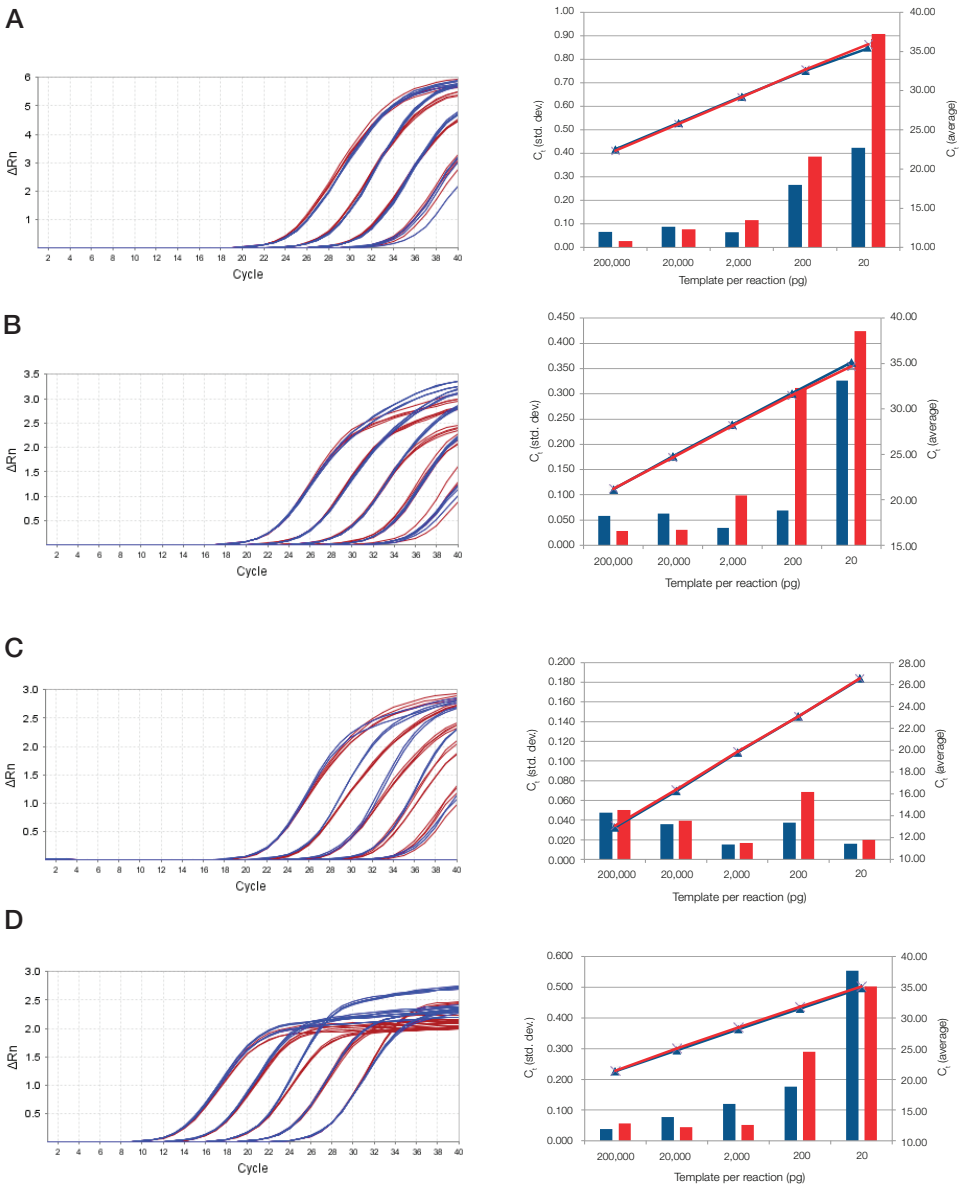

Figure 2. Comparison of singleplex and multiplex gene expression assays. (A) EGFR assay, FAM dye; **(B)** BRCA1 assay, VIC dye; **(C)** ESR1 assay, JUN dye; **(D)** RNase P assay, ABY dye. Amplification was performed on the QuantStudio 7 Real-Time PCR System using TaqMan Multiplex Master Mix. The figure shows amplification plots (left) and linear curves (right) for 4 assays amplified in singleplex (blue) and 4-plex reactions (red) in a dilution series from 20,000 pg to 2 pg of reference colon cDNA per 10 µL reaction. Average C_t value (lines) and average standard deviation (bars) for the dilution series are represented in their respective graphs and show the concordance between singleplex and 4-plex reactions. PCR efficiencies are: 96.09% for EGFR singleplex and 96.39% for EGFR 4-plex; 93.56% for BRCA1 singleplex and 94.93% for BRCA1 4-plex; 97.13% for ESR1 singleplex and 95.81% for ESR1 4-plex; 96.91% for RNase P singleplex and 98.1% for RNase P 4-plex.

Improved probe performance

Introduction of ABY and JUN reporter dyes and Mustang Purple passive reference dye allows for optimal 4-color multiplex assays when used with our FAM and VIC reporter dyes. Please note that ABY and JUN reporter dyes are available only with QSY quencher, while FAM and VIC dyes are available with either MGB or QSY quencher. A comparison with a set of dyes from another supplier shows that our combination of dyes provides an earlier C_t for the majority of assays (Figure 3).

Optimized multiplex master mix

In multiplex PCR, it's important to have a robust master mix that allows for amplification of each target in a highly competitive environment. Our new master mix composition was developed to provide optimal multiplex performance for each target in the reaction. A comparison of our master mix and a master mix from another supplier in a 4-plex reaction shows an earlier C_t for 3 of the targets amplified with our new master mix and a lower standard deviation for most of the dilution points, demonstrating the excellent performance of our solution (Figure 4).

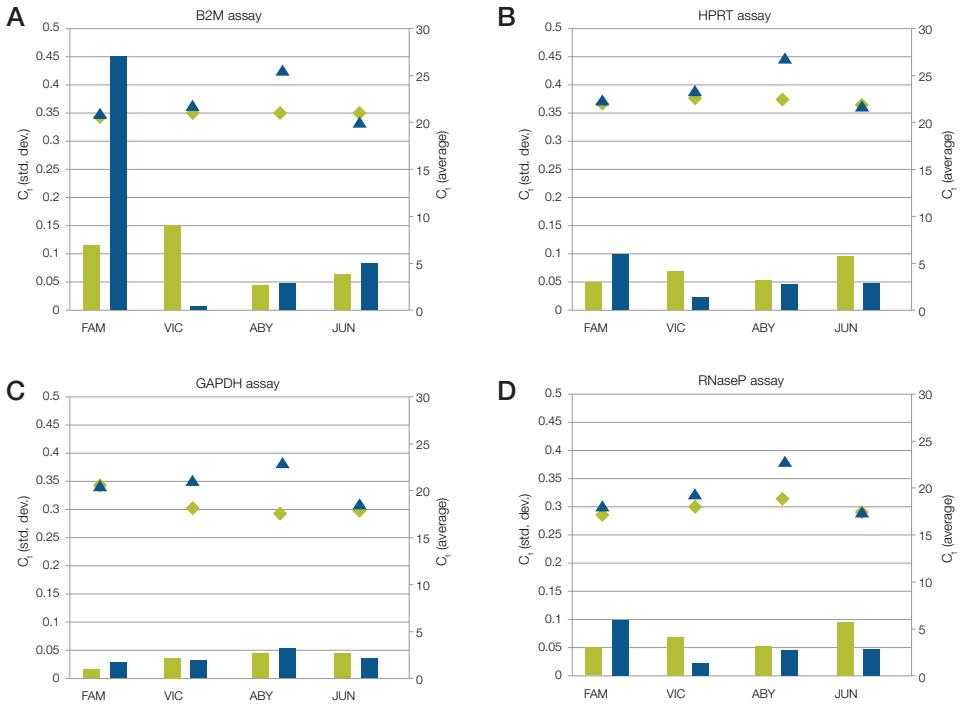

Figure 3. Comparison of our new dye combination with a dye combination from another supplier. Probes for **(A)** B2M, **(B)** HPRT, **(C)** GAPDH, and **(D)** RNase P gene expression assays were synthesized with FAM, VIC, ABY, and JUN dyes with QSY quencher (green bars and diamonds) and with another commercially available dye combination (blue bars and triangles). All possible gene-dye combinations were tested. Reactions were prepared with TaqMan Multiplex Master Mix using 900 nM of primer, 250 nM of probe, and 10 ng of cDNA. Amplification was performed on the QuantStudio 7 Real-Time PCR System using TaqMan Multiplex Master Mix. Bars represent average standard deviation. Triangles and diamonds represent average C_t values.

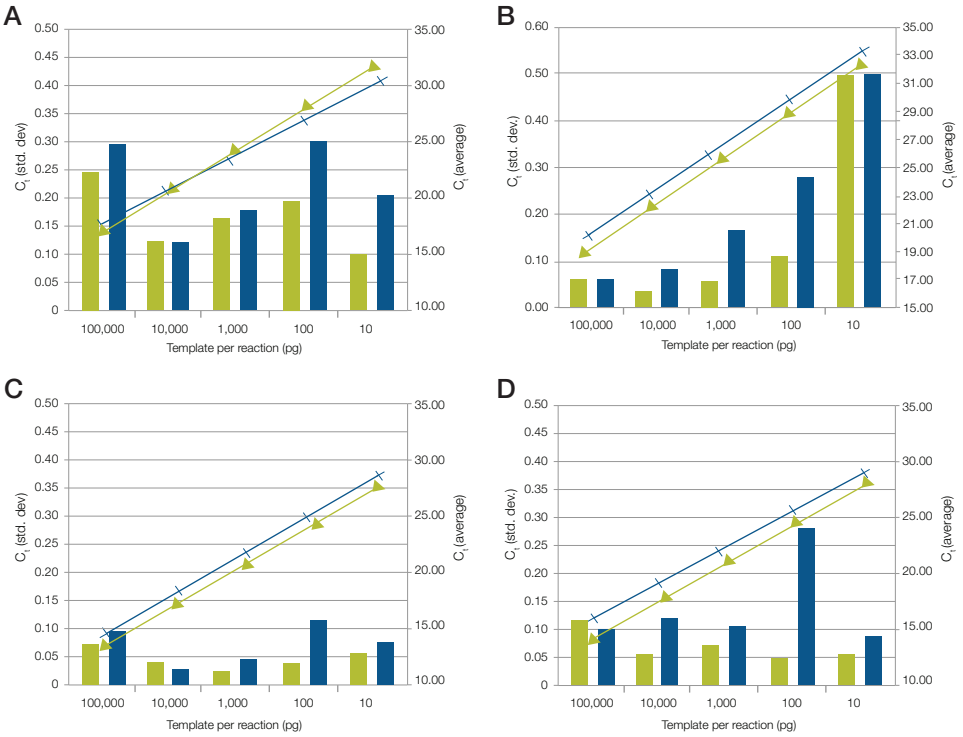

Figure 4. Comparison of TaqMan Multiplex Master Mix with another commercially available master mix. (A) B2M assay, FAM dye; **(B)** RNase P assay, VIC dye; **(C)** GAPDH assay, ABY dye; **(D)** HPRT assay, JUN dye. All assays used QSY quencher. The graph shows average standard deviation (bars) and average C_t values (cross and triangle) for 4-plex reactions using a dilution series from 100 ng to 10 pg of cDNA per 10 µL reaction. All amplifications were performed on the ViiA 7 Real-Time PCR System using the cycling conditions recommended for each master mix. Green represents TaqMan Multiplex Master Mix, and blue represents 4-plex reactions with another commercially available master mix.

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Optimized to minimize time-to-results

Developing a multiplex PCR assay requires time to correctly design the assay and optimize the reaction. Using our complete solution, for which all components were developed to work together, helps increase your chances of success and limits your development time. A new multiplex PCR user guide was developed to guide you through the development and optimization process [1], and our custom services will allow you to delegate assay design to our experienced team to minimize your efforts.

References

1. Multiplex PCR User Guide. Available at thermofisher.com/multiplexqpcr
2. TaqMan multiplex qPCR: Accurate, sensitive, and as efficient as traditional singleplex qPCR. Application note available at lifetechnologies.com/multiplexqpcr

Ordering information

Product	Cat. No.
TaqMan QSY probes	
TaqMan QSY Probe, 6,000 pmol	4482777
TaqMan QSY Probe, 20,000 pmol	4482778
TaqMan QSY Probe, 50,000 pmol	4482779
Control kits	
TaqMan GAPDH Assay, JUN-QSY 20X	4485712
TaqMan GAPDH Assay, JUN-QSY PL 20X	4485713
TaqMan RNaseP Assay, ABY-QSY 20X	4485714
TaqMan RNaseP Assay, ABY-QSY PL 20X	4485715
Multiplex master mixes	
TaqMan Multiplex Master Mix, 1 mL	4461881
TaqMan Multiplex Master Mix, 5 mL	4461882
TaqMan Multiplex Master Mix, 50 mL	4486295

Other formats are available at **lifetechnologies.com/multiplexqpcr**

Calibration plates	
96-Well Calibration Plate, Mustang Purple dye	4461599
96-Well Calibration Plate, JUN dye	A24737
96-Well Calibration Plate, ABY dye	A24738

Calibration plates are also available for 96-well Fast and 384-well plate formats.
Visit **thermofisher.com/multiplexqpcr** for more information.

Find out more at **thermofisher.com/multiplexqpcr**

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TaqMan™ Assay Multiplex PCR Optimization

APPLICATION GUIDE

for use with:

TaqMan™ Gene Expression Assays

TaqMan™ SNP Genotyping Assays

TaqMan™ MicroRNA Assays

TaqMan™ Advanced miRNA Assays

Publication Number MAN0010189

Revision C.0

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

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Revision history: Pub. No. MAN0010189

Revision	Date	Description
C.0	18 January 2022	• Added TaqMan™ MicroRNA Assays and TaqMan™ Advanced miRNA Assays. • Removed TaqMan™ Copy Number Assays. • Updated compatible Master Mixes, real-time PCR instruments, and calibration plates. • Updated for general style, formatting, and branding.
B.0	17 May 2014	Baseline for this revision history.

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Contents

1 Overview

About this guide

This guide is designed to give guidance to researchers for performing multiplex PCR with TaqMan™ Assays. It covers the following items:

- Guidance on optimizing multiplex PCR reactions
- Information to facilitate the performance of multiplex PCR and decrease the amount of time required to perform optimization
- Guidance on verification of multiplex PCR reaction performance
- Performing multiplex PCR with up to four probes

Overview of multiplex PCR

Multiplex PCR is the simultaneous amplification of multiple targets in a single reaction tube. Different reporters with distinct fluorescence spectra are used in multiplex PCR to track each individual amplification reaction. The real-time PCR instrument detects a signal from each reporter dye and determines the amount of each target.

Multiplex PCR can reduce the amount of sample that is required for real-time PCR because it measures more than one target in a single reaction. It also can reduce reaction costs compared to performing 4 singleplex reactions independently.

Multiplex PCR has the following additional advantages:

- It reduces the quantity of reagents required.
- It reduces the time that is required to set up experiments and analyze results.
- It improves precision by reducing the potential for pipetting errors.

Applications for multiplex PCR

The simplest application for multiplexing is to amplify one target gene and one endogenous control in a shared reaction well, using probes labeled with two distinct fluorescent dyes and the same pool of enzymes, nucleotides, and other reagents.

Multiplex PCR with multiple reporter dyes in one well is best suited for non-quantitative applications, such as genotyping and pathogen detection. For quantitative reactions, higher throughput formats such as 384–well plates and TaqMan™ OpenArray™ Plates provide an alternative methodology (spatial multiplexing). The disadvantages of spatial multiplexing, where the sample is divided into smaller reactions, is that the sensitivity of the assay is reduced.

Optimization and verification of multiplex PCR

Multiplex PCR is as sensitive and as accurate as amplifying a single gene, but it is more technically complex. Multiplex PCR conditions must be optimized, and it is necessary to verify the multiplex PCR.

The following factors can affect the reliability of multiplex PCR assays:

- Competition or inhibition between assays through interactions among the various primer pairs, probes, targets, amplicons, or any combination of these factors
- The relative expression levels of targets, including endogenous controls, and the range of their expression
- The efficiency of the multiplex assays
- Primer concentration affected by polymerase saturation

Thermo Fisher Scientific products for multiplex PCR

Thermo Fisher Scientific offers a complete multiplex PCR solution using TaqMan™ predesigned or custom assays that enables up to a 4-plex reaction for gene expression and a duplex reaction for genotyping analysis. The solution includes the following products, all of which are described in this guide.

- Up to four dyes optimized to work together with minimal spectral overlap
- TaqMan™ custom probes with a QSY quencher
- A set of master mixes optimized for multiplex PCR
- Spectral calibration plates designed for multiplex PCR

These, combined with our custom services and real-time PCR instruments and software, decrease the amount of time required to optimize your multiplex PCR. Optimization of reaction components such as magnesium is typically not necessary.

Overview of TaqMan™ Assays

Predesigned TaqMan™ Assay primer and probe sets are available for gene expression, genotyping, and microRNA analysis.

Table 2 Reporter and quencher dye options for predesigned TaqMan™ Assays

Assay type	Reporter dye options	Quencher dye
TaqMan™ Gene Expression Assays, predesigned	• FAM™ dye • VIC™ dye	Non-fluorescent MGB
TaqMan™ SNP Genotyping Assays, predesigned		
TaqMan™ MicroRNA Assays	FAM™ dye	Non-fluorescent MGB
TaqMan™ Advanced miRNA Assays		

Custom versions of the assays with VIC™ reporter dye and a non-fluorescent MGB quencher can be ordered.

A multiplex reaction should be limited to two MGB probes to avoid possible inhibition of PCR by MGB. Custom assays with different dyes and quenchers are required for more targets.

Overview of reporter dyes and quenchers

The sequence, the reporter dye, and the quencher must be appropriate for the probe used in the assay. The quencher should be capable of quenching the reporter dye. The absorbance range of the quencher must overlap sufficiently with the emission range of the reporter dye.

The choice of quencher can affect the Tm of the probe and therefore its compatibility with the real-time PCR experiment. For example, a probe sequence paired with an MGB quencher has a higher Tm than the same sequence paired with a QSY™ quencher. It is important to consider quencher choice when designing probes and calculating Tm. For questions about reporter dye and quencher compatibility, contact Technical Support.

For gene expression assays and genotyping assays, ABY™ dye and JUN™ dye are recommended with the QSY™ non–fluorescent quencher. Assays using the ABY™ dye and the JUN™ dye need to be designed with a longer probe (compared to MGB–quenched probes) to accommodate the QSY™ quencher.

MicroRNA assays are predesigned with a FAM™ reporter dye and non-fluorescent MGB quencher. Due to the short length of the microRNA, longer additional probes are not recommended, and multiplex reactions are limited to duplex PCR.

TaqMan™ QSY™ Probes

QSY™ probe non-fluorescent quencher can be used for designing Custom TaqMan™ QSY™ Probes. QSY™ custom probes can be substituted for the 3' TAMRA™ probe, the 3' BHQ™–1 probe, and some BHQ™–2 probes without redesigning the probe sequence. These probes will maintain the same Tm. MGB probes must be redesigned to accommodate a QSY™ quencher.

Custom TaqMan™ QSY™ Probes are available with FAM™ dye, VIC™ dye, ABY™ dye and JUN™ dye.

Table 3 Absorbance and emission wavelengths for recommended dyes

Dye[1]	Absorbance	Emission
FAM™ dye	496 nm	520 nm
VIC™ dye	532 nm	552 nm
ABY™ dye	568 nm	583 nm
JUN™ dye	606 nm	618 nm

[1] Other dyes can function in multiplex PCR reactions but the dyes listed in this table are recommended.

These dyes are optimized to work together for performing multiplex PCR on the following instruments:

- QuantStudio™ Real-Time PCR Systems
- ViiA™ 7 Real-Time PCR Systems
- 7500/7500 Fast Real-Time PCR Systems

They can be used to detect up to the following number of targets:

- Two loci with 2 alleles each in TaqMan™ Gene Expression Assays
- Two SNPs in TaqMan™ SNP Genotyping Assays

Table 4 TaqMan™ QSY™ Probes

Cat. No.	Amount
4482777	6,000 pmol
4482778	20,000 pmol
4482779	50,000 pmol

Master mixes for multiplex PCR

When assays are amplified together, they compete for the same reagents, including dNTPs, Mg^{2+}, and polymerase. The more targets that are assayed in a multiplex reaction, the more likely it is that the assays will compete for reagents and be inhibited. A master mix that is developed for specific multiplex PCR assays is recommended.

Note: The master mixes listed in the table below all contain heat-labile UNG.

Master Mix	Assays	Template	Passive reference dye	Inhibitor tolerance	Pre–PCR stability	Cycling mode
TaqMan™ Multiplex Master Mix	Gene expression	DNA or cDNA	MUSTANG PURPLE™ passive reference dye[1]	Standard	16 hours	Standard or fast
TaqPath™ ProAmp™ Multiplex Master Mix	Genotyping	DNA or cDNA	MUSTANG PURPLE™ passive reference dye[1]	Compatible with samples prepared from human or animal sources (buccal swabs, blood, and card punches)	72 hours	
TaqPath™ 1-Step Multiplex Master Mix	Gene expression Pathogen detection	RNA	Available without a passive reference dye or with MUSTANG PURPLE™ passive reference dye[1]	Tolerant of inhibitors commonly found in clinical samples	Use immediately	

[1] MUSTANG PURPLE™ dye is not compatible with StepOne™and StepOnePlus™ instruments, 7300 Real-Time PCR System, or QuantStudio™ 3 Real-Time PCR System.

TaqMan™ Multiplex Master Mix

TaqMan™ Multiplex Master Mix is formulated with optimized buffer components to accommodate multiplex amplification of up to four targets in a single reaction. It is supplied in a 2X concentration premix to perform real-time PCR using TaqMan™ Probes.

The master mix contains the following components:

- AmpliTaq™ Fast DNA Polymerase, UP (Ultra Pure)
- Heat-labile Uracil-DNA Glycosylase (UDG or UNG)
- MUSTANG PURPLE™ passive reference dye
- dNTP blend containing dUTP/dTTP

For more information, see *TaqMan™ Multiplex Master Mix Quick Reference* (Pub. No. MAN0009605).

Table 5 TaqMan™ Multiplex Master Mix

Cat. No.	Amount	Number of 20–µL reactions	Storage
4461881	1 mL	100	2–8°C
4461882	5 mL	500	
4461884	2 × 5 mL	1000	
4484262	5 × 5 mL	2500	
4484263	10 × 5 mL	5000	
4486295	50 mL	5000	

TaqPath™ ProAmp™ Multiplex Master Mix

TaqPath™ ProAmp™ Multiplex Master Mix is used for high-throughput genotyping experiments that require accurate results from samples containing PCR inhibitors. It is designed to deliver sensitive and reproducible results from genomic DNA targets. It is compatible with multiplex PCR for up to 4 targets.

The master mix contains the following components:

- Dual-Lock™ Taq DNA Polymerase
- Heat-labile uracil-DNA glycosylase (UDG or UNG)
- dNTPs with dUTP
- MUSTANG PURPLE™ passive reference dye
- Optimized buffer components

For more information, see *TaqPath™ ProAmp™ Master Mixes User Guide* (Pub. No. MAN0015758).

Table 6 TaqPath™ ProAmp™ Multiplex Master Mix

Cat. No.	Amount	Number of 20–µL reactions	Storage
A30868	1 mL	100	2–8°C
A30869	1 x 10 mL	1000	
A30870	1 x 50 mL	5000	
A30873	2 x 10 mL	2000	
A30874	2 x 50 mL	10,000	

TaqPath™ 1-Step Multiplex Master Mix

TaqPath™ 1-Step Multiplex Master Mix allows cDNA synthesis and PCR amplification in the same well. This minimizes pipetting and hands-on time.

The master mix is available in two formulations.

- TaqPath™ 1-Step Multiplex Master Mix (No ROX™)—Does not contain a passive reference dye
- TaqPath™ 1-Step Multiplex Master Mix—Contains MUSTANG PURPLE™ passive reference dye

Both formulations of the master mix contain the following components:

- Thermostable MMLV reverse transcriptase
- Fast DNA polymerase
- Heat-labile uracil-DNA glycosylase (UDG or UNG)
- dNTPs

Both versions of the master mix have the following benefits:

- High sensitivity to detect low-copy targets with reproducible C_t results
- Six logs of dynamic range with both RNA and DNA targets
- Multiplex PCR of up to 4 targets simultaneously with or without a passive reference dye
- Tolerance of inhibitors commonly found in clinical samples

For more information, see *TaqPath™ 1-Step Multiplex Master Mix User Guide* (Pub. No. MAN0014269).

Table 7 TaqPath™ 1-Step Multiplex Master Mix (No ROX™)

Cat. No.	Amount	Number of 20–µL reactions	Storage
A28521	0.5 mL	50	2–8°C
A28522	5 x 1 mL	500	
A28523	10 mL	1000	

Table 8 TaqPath™ 1-Step Multiplex Master Mix

Cat. No.	Amount	Number of 20–µL reactions	Storage
A28525	0.5 mL	50	2–8°C
A28526	5 x 1 mL	500	
A28527	10 mL	1000	

MUSTANG PURPLE™ passive reference dye

The MUSTANG PURPLE™ passive reference dye has an absorption wavelength of 647 nm and an emission wavelength of 654 nm. It provides an internal reference for normalizing the reporter-dye signal during data analysis. Normalization corrects for fluctuations in fluorescence due to changes in concentration or volume.

MUSTANG PURPLE™ dye is used in place of the typical ROX™ passive reference dye to allow the use of JUN™ dye. ROX™ dye and JUN™ dye are detected with the same filter.

Required materials not supplied

Real-time PCR instruments

The following real-time PCR instruments can be used for multiplex PCR:

- QuantStudio™ 3 and 5 Real-Time PCR Systems
- QuantStudio™ 6 / QuantStudio™ 7 Flex System
- QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems
- QuantStudio™ 12K Flex Real–Time PCR System
- ViiA™ 7 Real-Time PCR System
- 7500 Real-Time PCR System
- 7500 Fast Real-Time PCR System

  Note: The software for the 7500 Fast Real-Time PCR System does not support assigning two SNP assays to one well, but analysis can be done with the Genotyping application on Thermo Fisher™ Connect.

- StepOne™ and StepOnePlus™ Real-Time PCR Systems

  Note: The StepOne™ Real-Time PCR System has three filters, which permits it to run a duplex with FAM™ dye and VIC™ dye, and use a master mix that contains ROX™ as the passive reference dye. The StepOnePlus™ Real-Time PCR System has four filters and can be used for three dyes with ROX™ as the passive reference dye. Neither system supports assigning two SNP assays to one well.

IMPORTANT! Do not use the StepOne™ Real-Time PCR System and StepOnePlus™ Real-Time PCR System for more than two concurrent assays.

Filter selection

Table 9 Filters for QuantStudio™ 3 and 5 Real-Time PCR Systems

Dye	System and filter	
	QuantStudio™ 3 System	QuantStudio™ 5 System
FAM™ dye	x1m1	x1m1
VIC™ dye	x2m2	x2m2
ABY™ dye	x3m3	x3m3
JUN™ dye[1]	x4m4	x4m4
MUSTANG PURPLE™ dye	N/A	x5m5

[1] Used as a passive reference dye.

Table 10 Filters for ViiA™ 7 Real-Time PCR System, QuantStudio™ 6 and 7 Flex Real-Time PCR Systems, QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems, and QuantStudio™ 12K Flex Real–Time PCR System

Dye	System and filter			
	ViiA™ 7 Real-Time PCR System	QuantStudio™ 6 Flex System and QuantStudio™ 6 Pro System	QuantStudio™ 7 Flex System and QuantStudio™ 7 Pro System	QuantStudio™ 12K Flex Real–Time PCR System
FAM™ dye	x1m1	x1m1	x1m1	x1m1
VIC™ dye	x2m2	x2m2	x2m2	x2m2
ABY™ dye	x3m3	x3m3	x3m3	x3m3
JUN™ dye	x4m4	x4m4	x4m4	x4m4
MUSTANG PURPLE™ dye	x5m5	x5m5	x5m5	x5m5
—[1]	x6m6	N/A	x6m6	x6m6

[1] Contact Technical Support.

Table 11 Filters for 7500 Real-Time PCR System and 7500 Fast Real-Time PCR System

Dye	System and filter	
	7500 Real-Time PCR System	7500 Fast Real-Time PCR System
FAM™ dye	Filter A	Filter A
VIC™ dye	Filter B	Filter B
ABY™ dye	Filter C	Filter C
JUN™ dye	Filter D	Filter D
MUSTANG PURPLE™ dye	Filter E	Filter E

Table 12 Filters for StepOne™ and StepOnePlus™ Real-Time PCR Systems

Dye	System and filter	
	StepOne™ Real-Time PCR System	StepOnePlus™ Real-Time PCR System
FAM™ dye	Filter 1	Filter 1
VIC™ dye	Filter 2	Filter 2
ABY™ dye	N/A	Filter 3
ROX™ dye	Filter 3	Filter 4

Spectral calibration plates

It is recommended that calibration and verification of your real-time instrument be performed as outlined in the instrument user guide.

For spectral calibration on the 7500 Real-Time PCR System or the 7500 Fast Real-Time PCR System, set up custom calibration files for FAM™ dye and VIC™ dye plates using new file names.

IMPORTANT! Do not overwrite the original system FAM™ dye and VIC™ dye calibration files. See your instrument user guide.

Calibration Plate	Source	Real-Time PCR System		
		7500 Real-Time PCR System	7500 Fast Real-Time PCR System	QuantStudio™ 6 and 7 Flex Real-Time PCR Systems and QuantStudio™ 12K Flex Real–Time PCR System[1]
Standard 96-well plates (0.2 mL)				
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, 96-well	A24738	✓	–	✓
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, 96-well	A24737	✓	–	✓
FAM™ Dye Spectral Calibration Plate, 96-well	4432327	✓	–	✓
VIC™ Dye Spectral Calibration Plate, 96-well	4432334	✓	–	✓
MUSTANG PURPLE™ Dye Spectral Calibration Plate for Multiplex qPCR, 96-well	4461599	✓	–	✓
Fast 96-well plates (0.1 mL)				
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	A24734	–	✓	✓
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	A24735	–	✓	✓

(continued)

Calibration Plate	Source	Real-Time PCR System		
		7500 Real-Time PCR System	7500 Fast Real-Time PCR System	QuantStudio™ 6 and 7 Flex Real-Time PCR Systems and QuantStudio™ 12K Flex Real–Time PCR System[1]
FAM™ Dye Spectral Calibration Plate, Fast 96-well	4432389	–	✓	✓
VIC™ Dye Spectral Calibration Plate, Fast 96-well	4432396	–	✓	✓
MUSTANG PURPLE™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	4457328	–	✓	✓
384-well plates				
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, 384-well	A24736	–	–	✓
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, 384-well	A24733	–	–	✓
FAM™ Dye Spectral Calibration Plate, 384-well	4432271	–	–	✓
VIC™ Dye Spectral Calibration Plate, 384-well	4432278	–	–	✓
MUSTANG PURPLE™ Dye Spectral Calibration Plate for Multiplex qPCR, 384-well	4457334	–	–	✓

[1] The instrument must be configured with the appropriate block.

Calibration Plate	Source	Real-Time PCR System	
		QuantStudio™ 3 and 5 Real-Time PCR Systems[1]	QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems
Standard 96-well plates (0.2 mL)			
QuantStudio™ 3/5 Spectral Calibration Plate 1 (FAM™, VIC™, ROX™, and SYBR™ dyes), 96-Well 0.2-mL	A26331	✓	✓
QuantStudio™ 3/5 Spectral Calibration Plate 2, 96-Well 0.2-mL (ABY™, JUN™, and MUSTANG PURPLE™ dyes)	A26332	✓	✓
QuantStudio™ 3/5 Spectral Calibration Plate 3, 96-Well 0.2-mL (TAMRA™, NED™, and Cy™5 dyes)	A26333	✓	✓
Fast 96-well plates (0.1 mL)			
QuantStudio™ 3/5 Spectral Calibration Plate 1, (FAM™, VIC™, ROX™, and SYBR™ dyes), 96-well, 0.1 mL	A26336	✓	–
QuantStudio™ 3/5 Spectral Calibration Plate 2 (ABY™, JUN™, MUSTANG PURPLE™ dyes), 96-well Fast (0.1-mL) Plate	A26337	✓	–
QuantStudio™ 3/5 Spectral Calibration Plate 3 (TAMRA™, NED™ and Cy™5 dyes) 96-well Fast (0.1-mL) Plate	A26340	✓	–

(continued)

Calibration Plate	Source	Real-Time PCR System	
		QuantStudio™ 3 and 5 Real-Time PCR Systems[1]	QuantStudio™ 6 Pro and 7 Pro Real-Time PCR Systems
384-well plates			
QuantStudio™ 5 Spectral Calibration Plate 1, (FAM™, VIC™, ROX™ TAMRA™, and SYBR™ dyes), 384-well	A26334	✓	–
QuantStudio™ 5 Spectral Calibration Plate 2 (ABY™, JUN™, MUSTANG PURPLE™, NED™, and Cy™5 dyes), 384-well	A26335	✓	–

[1] The instrument must be configured with the appropriate block.

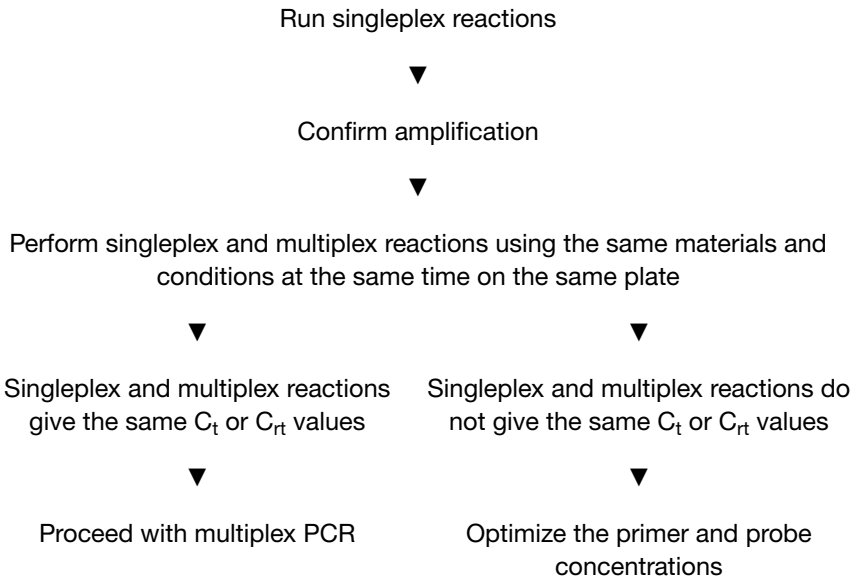
Other materials and equipment not supplied

For compatible instruments, see “Real-time PCR instruments” on page 15.

Table 13 Other materials and equipment required for the workflow

Item	Source
Software	
Primer Express™ Software	4363991
TaqMan™ Genotyper Software	**thermofisher.com/qpcrsoftware**
Equipment	
Centrifuge, with adapter for 96-well plates or adapter for 384-well plates	MLS
Microcentrifuge	MLS
Vortex mixer	MLS
Pipettes	MLS
Master Mixes	
TaqMan™ Multiplex Master Mix	4461882
TaqPath™ ProAmp™ Multiplex Master Mix	A30870
TaqPath™ 1-Step Multiplex Master Mix	A28526
Tubes, plates, and other consumables	
Tubes, plates, and film	**thermofisher.com/plastics**
Aerosol-resistant barrier pipette tips	MLS
Disposable gloves	MLS
Reagents	
Nuclease-free water	AM9930
TE, pH 8.0, RNase-free	AM9849

Workflow



Note: For gene expression assays, it is acceptable for the C_t or C_{rt} values to be within one cycle of each other.

2 Overview of component selection for multiplex PCR

Primer design

We recommend starting with predesigned TaqMan™ Assay primer and probe sets, which are optimized for reaction components such as magnesium. A bioinformatics check should be done on TaqMan™ Assays that are intended for multiplexing.

If a custom-designed assay is needed, use the following guidelines.

- Primer design should include bioinformatics analysis to minimize non-target binding to the template DNA, to the probe, or to each other.
- The Tm of each PCR primer should be between 58–60°C, and the Tm of both primers to be used in the reaction should ideally be within 1–2°C of each other.
- For more information, see “Guidelines for primer design” on page 49.

Note: We recommend using Primer Express™ Software to calculate the Tm the primers and the Tm of the probe. Alternatively, use the Custom Assay Design Tool, available at **Assay Design Tool**. Other primer–design software may provide different Tm values due to different assumptions in the algorithm.

Probe design

- For gene expression experiments, the Tm of probes should be approximately 10°C higher than the Tm of the primers (approximately 68–70°C).
- For SNP genotyping, the Tm of the probes should be approximately 65–67°C and ideally within 1°C of each other.
- MGB probe sequences are not interchangeable with TaqMan™ QSY™ Probe sequences because the TaqMan™ QSY™ Probe length will be too short.
- For custom–designed probes, see *Primer Express™ Software Version 3.0 Getting Started Guide* (Pub. No. 4362460).
- To have predesigned assays redesigned, email **specialty_oligos@thermofisher.com**. Include the ID of the assay that you want redesigned from an MGB probe to a QSY probe. You will receive ordering instructions for the QSY version of the assay.

IMPORTANT! We recommend verification of the multiplex assay. Use samples that are representative of your experiment and ensure that the dynamic range of your multiplex assay meets the needs of your experiment. Contact Technical Support for assistance with validation.

Primer and probe selection

The guidelines and tools described in this section are for designing assays. They are not applicable to pre-designed assays.

- A multiplex reaction can contain up to eight primers and four probes (to produce four amplicons). It is good practice to minimize conditions that result in primer-dimer formation or other unfavorable interactions by optimizing primer concentrations and avoiding complementary sequences.
- We recommend avoiding degenerate oligos. It is preferable synthesize each oligo separately and mix them together in equal proportions.
- Ensure that amplicons do not overlap. If the amplicon coordinates are not known, map genomic assays to the genome, or gene expression assays to the transcriptome.
- Use the following tools to verify coordinates:
  - UCSC *In Silico* PCR tool: **genome.ucsc.edu/cgi-bin/hgPcr**
  - NCBI Basic Local Alignment Search Tool: **blast.ncbi.nlm.nih.gov/Blast.cgi**
- Ensure that amplicons are all approximately the same size, generally less than 150 bp, and that primer and probe dimers do not form (across all primer pairs).
- Use the Multiple Primer Analyzer tool at **thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html**.

Probe selection

- You can use predesigned assays containing FAM™ dye– or VIC™ dye–labeled MGB–NFQ probes (also referred to as MGB probes) in multiplex reactions. However, the multiplex reaction should not contain more than two MGB probes to ensure successful amplification.
- For the third and fourth dye, we recommend using ABY™ dye– and JUN™ dye–labeled probes made with QSY™ quencher. Preformulated assays made with ABY™ probes and JUN™ probes are available with QSY™ quenchers from **specialty_oligos@thermofisher.com**.
- Multiplex gene expression analysis can use gene expression assays with FAM™ dye and VIC™ dye combined with one assay containing a custom ABY™–QSY™ probe and one assay containing a custom JUN™–QSY™ probe.
- For multiplex SNP genotyping analysis, an existing SNP genotyping assay with FAM™dye and VIC™ dye can be combined with a custom ABY™–QSY™/JUN™–QSY™ assay.
- MicroRNA assays can be duplexed using a FAM™ dye– and VIC™ dye–labeled assay with MGB quencher. The VIC™ dye–labeled assay can be ordered through **specialty_oligos@thermofisher.com**.
- Custom probes can be ordered to help with concentration optimization. They can be ordered with MGB-NFQ quenchers and QSY™ quenchers. Order at **www.thermofisher.com/taqman-primers-probes**.
- Probes with FAM™ dye and VIC™ dye can be made with either a QSY™ quencher or an MGB quencher. Changing the quenchers requires a redesign of the probe to maintain the correct Tm. ABY™ probes and JUN™ probes can be made with a QSY™ quencher.

Dye selection

- Choose dyes that are compatible with the instrument and the other dyes used for the multiplex PCR. See the appropriate instrument user guide for dye compatibility.
- Match dye intensity with target abundance by pairing the brightest dye with low abundance targets, and the dimmest dye with high abundance targets (for example, an internal positive control).

PCR reaction buffer

- All of the assays are amplified in the same tube. They compete for the same reagents (dNTPs, Mg^{2+}, and polymerase).
- The more targets that are assayed in a multiplex reaction, the more likely it is that there will be competition for reagents and inhibition between assays.
- Multiplex Master Mixes are recommended to offset the effect of competition for reagents (see “Other materials and equipment not supplied” on page 22).

Instrument calibration

- Calibrate the real–time instrument for the dyes being used in the assay.
- The instrument must be calibrated for MUSTANG PURPLE™ dye if it is used as the passive reference dye.
- Using more dyes in a reaction well increases the importance of high-quality spectral calibration.

Passive reference dye

Do not use the default passive reference setting (ROX™ dye) when using MUSTANG PURPLE™ dye as a passive reference. They are read in different channels. Ensure that the passive reference is set to MUSTANG PURPLE™ dye when it is used as a passive reference. After adding a MUSTANG PURPLE™ custom dye calibration, it will appear as an option in the menu. For information on the calibration process, refer to the appropriate guide for your instrument.

Assignment of reporter dyes

IMPORTANT! It is critical to calibrate the instrument for the dyes used in the experiment and to configure the instrument with the correct dyes in each well. If dyes are not assigned to their wells or dyes that are not used are assigned, the analysis will not be valid.

Label the wells in the plate with the appropriate dyes because individual dye components overlap with adjacent dye channels.

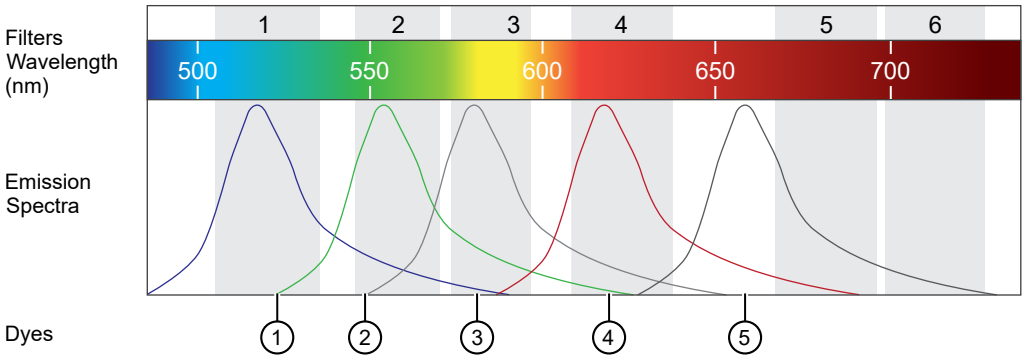


Figure 1 Overlapping reporter dye spectra

① FAM™ dye (approximate peak emission 517 nm)
② VIC™ dye (approximate peak emission 551 nm)
③ ABY™ dye (approximate peak emission 580 nm)
④ JUN™ dye (approximate peak emission 617 nm)
⑤ MUSTANG PURPLE™ dye (approximate peak emission 654 nm)

Overlapping spectra from each dye in a well and across all filters generate the composite spectrum that represents a raw data fluorescent reading. The instrument distinguishes the contribution of each dye to the raw spectral data collected. This process is called multicomponenting. Refer to your instrument user guide to determine which dyes your instrument detects.

The instrument compares the raw spectra with a set of pure dye standards contained in the calibration file. When a plate document is saved after data analysis, the software stores the pure spectra information with the rest of the collected fluorescent data for that experiment.

After calibration of the instrument for each dye, the instrument can subtract the background from the dye that bleeds into the other dye. For example, the spectra for VIC™ dye and FAM™ dye overlap to some extent. If a probe with FAM™ dye is in the well and the software label is VIC™ dye, the instrument will read the fluorescence of the probe as VIC™ dye, which usually gives a value (though weaker than a FAM™ dye value). This is normal. On an instrument calibrated for both dyes, the algorithm accounts for the overlap.

The instruments allow the user to reassign the wells after the run is complete, provided that the data has been collected for the correct filter.

3 Gene expression analysis

Overview of gene expression analysis

Gene expression analysis is performed to determine the relative expression levels between different gene targets within a single sample. Normalization between different samples is achieved by using a reference gene, typically an abundant housekeeping gene such as β-actin or 18S rRNA. In gene expression multiplexing experiments, the goal is to minimize the difference between the C_t value in singleplex and multiplex reactions.

TaqMan™ Gene Expression Assaysare easily duplexed, because both predesigned and custom assays are available with either FAM™ or VIC™ fluorescent reporter dyes. The most common duplex reaction is combining the target of interest and endogenous control assays in the same well. An endogenous control gene or set of genes is typically used to control for experimental variability due to RNA input and shows expression levels that are relatively constant and moderately abundant across tissues, cell types, and treatment protocols. For targets that are highly expressed (for example, 18S rRNA, a common endogenous control), we recommend choosing the VIC-primer-limited (VIC_PL) assay format, which has a decreased amount of primers to limit the housekeeping gene from consuming the qPCR reagents before the target of interest can amplify.

See *TaqMan™ Gene Expression Assays User Guide—single-tube assays* (Pub. No. 4333458) and *Introduction to Gene Expression Getting Started Guide* (Pub. No. 4454239).

Up to four targets can be multiplexed in a single reaction, depending on the probes that are selected and the number of filters available on the real-time PCR instrument. In this case, optimization of primers and probe for each target is required.

Procedural guidelines

Target abundance

The amount of target and endogenous control in a sample can affect the outcome of PCR results when performing multiplex assays. Abundance can be characterized by the C_t range.

Table 14 Target expression levels

Target expression level	C_t range
High	$C_t \leq 20$
Medium	$20 < C_t \leq 27$
Low	$27 < C_t \leq 35$
No template control	$C_t > 35$

Recommendations for target abundance

Different methods are recommended to offset the effects of target abundance when optimizing multiplex assays.

Target properties	Recommendations
Some targets more abundant than others	• When multiplex PCR is performed on a sample in which one or more targets is more abundant than the others, the assays for the abundant species should be primer–limited. See “Primer and probe concentration” on page 31. • Housekeeping genes and endogenous controls are highly expressed. • Using primer-limited reaction conditions prevents consumption of reactants (dNTPs) before the less abundant target is amplified, by forcing the PCR reaction to plateau due to the lack of primers. • For very highly abundant transcripts or especially bright assays, probe concentration might need to be adjusted. See “Guidelines for primer design” on page 49.
Targets are of similar abundance	• When all targets are present in approximately equal abundance, no single assay needs to be primer-limited. • Assay optimization is recommended to minimize the C_t difference between single and multiplex reactions. • Start with 900 nM for each primer and 250 nM for the probe in the final reaction mix.
Either target may be more abundant	• If any of the targets could be more abundant than the others, all assays need to be primer-limited. • Establishing reaction conditions for extreme cases of low or high abundance is suggested for optimization.

Primer and probe concentration

Optimization of the concentrations of primers and probe for each target is an important first step in assembling a three- or four-color reaction. The same considerations apply for both 1-step and 2-step RT-PCR.

In multiplex reactions, start with a standard condition. Optimization of the assay may be necessary.

- Forward primer—900 nM
- Reverse primer—900 nM
- Probe—250 nM

If one or more targets are more abundant than the others, the assays should be primer-limited. Primer-limited TaqMan™ Assays have a final primer concentration of 150 nM each with 250 nM probe concentration. This is a suggested starting point to optimize for abundant targets.

If the required endogenous control target is available as a primer-limited assay, begin by verifying your duplex PCR. If not, you must limit the primer concentration in the assay. The goal of limiting the primer concentration in the assay is to find the primer concentration that gives the lowest (earliest) possible C_t value for the more abundant target without distorting the C_t value of the less abundant target. Limiting the primer concentration for the more abundant target has the effect of lowering its ΔRn. However, the C_t should remain unchanged under primer-limited conditions. A sample should be assayed using decreasing amounts of primer in order to determine the optimal primer concentration for each assay.

Dye selection

Make dye and target assignments to balance fluorescence levels in the multiplex reaction.

- FAM™ dye and ABY™ dye can be used for targets with low to medium levels of expression.
- VIC™ dye and JUN™ dye can be used for targets with high levels of expression.

Probe selection

- Up to four targets can be multiplexed in a single reaction using TaqMan™ QSY™ Probes (FAM™ dye, VIC™ dye, ABY™ dye, and JUN™ dye).
- Use no more than two probes that contain the MGB group. To multiplex three or more gene expression assays, order assays designed with a non-MGB quencher (**specialty_oligos@thermofisher.com**).

Methods

Prepare singleplex PCR reactions

The goal of running singleplex PCR is to ensure the performance of the reaction components and PCR conditions. If you are using TaqMan™ Predesigned or Custom Assays, verification of performance under singleplex conditions is unnecessary. Predesigned and Custom are available as FAM™ or VIC™ assays. If you need to use ABY™ and JUN™ dye assays or custom probe and primers using any dye, follow this procedure.

Plan to run singleplex reactions in triplicate or quadruplicate.

1. Prepare an Assay Mix for FAM™ dye, VIC™ dye, ABY™ dye, and JUN™ dye, according to the expression level of the target.

Target expression level	Concentration			
	Assay Mix (Final)	Primer 1	Primer 2	Probe 1
High	20X	3 µM	3 µM	5 µM
Medium	20X	6 µM	6 µM	5 µM
Low	20X	18 µM	18 µM	5 µM

Note: Changes might be required for optimal performance.

With a 20X Assay Mix, the concentrations of the primers and probes in the reaction will be as follows:

- High target expression level
  - Primers—150 nM
  - Probes—250 nM
- Medium target expression level
  - Primers—300 nM
  - Probes—250 nM
- Low target expression level
  - Primers—900 nM
  - Probes—250 nM

2. Prepare PCR Reaction Mix for each assay in an appropriately-sized microcentrifuge tube, according to one of the following tables.

Table 15 96–well fast (0.1–mL) plate and 384–well (0.1–mL) plate

Component	Volume per reaction[1]			
	Singleplex reaction 1	Singleplex reaction 2	Singleplex reaction 3	Singleplex reaction 4
Master Mix	5 µL	5 µL	5 µL	5 µL
FAM™ dye Assay Mix (20X)	0.5 µL	—	—	—
VIC™ dye Assay Mix (20X)	—	0.5 µL	—	—
ABY™ dye Assay Mix (20X)	—	—	0.5 µL	—
JUN™ dye Assay Mix (20X)	—	—	—	0.5 µL
Nuclease–free water	3.5 µL	3.5 µL	3.5 µL	3.5 µL
Total PCR Reaction Mix volume	**9.0 µL**	**9.0 µL**	**9.0 µL**	**9.0 µL**

[1] Add 10% overage.

Table 16 96–well standard (0.2–mL) plate

Component	Volume per reaction[1]			
	Singleplex reaction 1	Singleplex reaction 2	Singleplex reaction 3	Singleplex reaction 4
Master Mix	10 µL	10 µL	10 µL	10 µL
FAM™ dye Assay Mix (20X)	1 µL	—	—	—
VIC™ dye Assay Mix (20X)	—	1 µL	—	—
ABY™ dye Assay Mix (20X)	—	—	1 µL	—
JUN™ dye Assay Mix (20X)	—	—	—	1 µL
Nuclease–free water	7 µL	7 µL	7 µL	7 µL
Total PCR Reaction Mix volume	**18 µL**	**18 µL**	**18 µL**	**18 µL**

[1] Add 10% overage.

3. Mix the components thoroughly, then centrifuge briefly to collect the contents at the bottom of each tube.

4. Transfer the appropriate volume of PCR Reaction Mix to each well of an optical reaction plate.
   - 96–well fast (0.1–mL) plate or 384–well (0.1–mL) plate: 9.0 µL
   - 96–well standard (0.2–mL) plate: 18 µL

5. Add cDNA template to each well.

   Include a no-template control with water in place of cDNA.
   - 96–well fast (0.1–mL) plate or 384–well (0.1–mL) plate: 1 µL (1–10 ng of cDNA)
   - 96–well standard (0.2–mL) plate: 2 µL (2–20 ng of cDNA)

   Note: The amount of cDNA is calculated based on the amount of RNA input from the RT reaction.

6. Seal the plate with an optical adhesive cover, vortex, then centrifuge briefly to collect the contents at the bottom of each well.

Set up and run the real-time PCR instrument

See the appropriate instrument user guide for detailed instructions to program the thermal–cycling conditions or to run the plate.

Note: For more information see "Related documentation and software" on page 58.

IMPORTANT! Assign only the targets present in each reaction well of the plate to the corresponding well. Do not assign all the targets to a well.

1. Select the cycling mode appropriate for the master mix.

   IMPORTANT! The cycling mode depends on the master mix that is used in the reaction. The cycling mode does not depend on a standard or fast plate format.

2. Set up the thermal protocol for your instrument.

Table 17 ViiA™ 7, and QuantStudio™ Real-Time PCR Systems

Step	Temperature	Time	Cycles
Enzyme activation	95°C	20 seconds	1
Denature[1]	95°C	1 second	40
Anneal / Extend[2]	60°C	20 seconds	

[1] Denature time can be increased if needed for long amplicons.
[2] In some cases, increasing the anneal/extend time has been found to improve the performance of multiplex reactions.

Table 18 7500/7500 Fast Real-Time PCR Systems

Step	Temperature	Time	Cycles
Enzyme activation	95°C	20 seconds	1
Denature[1]	95°C	3 seconds	40
Anneal / Extend[2]	60°C	30 seconds	

[1] Denature time can be increased if needed for long amplicons.
[2] In some cases, increasing the anneal/extend time has been found to improve performance of multiplex reactions.

3. Set the reaction volume appropriate for the reaction plate.
   - 96–well fast (0.1–mL) plate and 384–well (0.1–mL) plate: **10 µL**
   - 96–well standard (0.2–mL) plate: **20 µL**
4. Load the plate into the real–time PCR instrument.
5. Start the run.
6. Analyze results of the singleplex real-time PCR reactions.

   Use the Relative Quantification application on Thermo Fisher™ Connect to view the amplification curves.
   - Replicates on 96– or 384–well plates with C_t values less than 30 should not be more than one-half of a C_t value apart.
   - The amplification curves should be parallel to each other.

   Perform a detailed analysis after collecting the multiplex real–time PCR data.

Visual assessment of exponential PCR phase efficiency

The exponential phase of PCR is the first phase of PCR in which all the PCR reaction components are in excess and amplification efficiency is consistent. As PCR cycling progresses and the amplified product increases in amount, eventually the exponential phase transitions to linear phase in which amplification efficiency declines, caused by a limitation in one of the PCR reaction components.

For a number of reasons, it is important for all real-time PCR assays to have 100% exponential phase efficiency, which means the target sequence doubles each cycle. Thermo Fisher Scientific TaqMan™ Assays have 100% exponential phase efficiency when run with the recommended universal cycling and chemistry conditions.

Exponential phase efficiency can be quickly and easily determined by visually comparing exponential phase slopes of multiple single assays in real-time PCR amplification plots using a log fluorescence (ΔRn) y-axis scale. See Figure 2. Parallel inter-assay exponential phase slopes indicate equivalent efficiencies, which equates to 100% efficiency, because assays with poor efficiency (<100%) have random efficiencies. Alternatively, exponential phase slopes of one assay can be compared to those from another assay with known 100% efficiency.

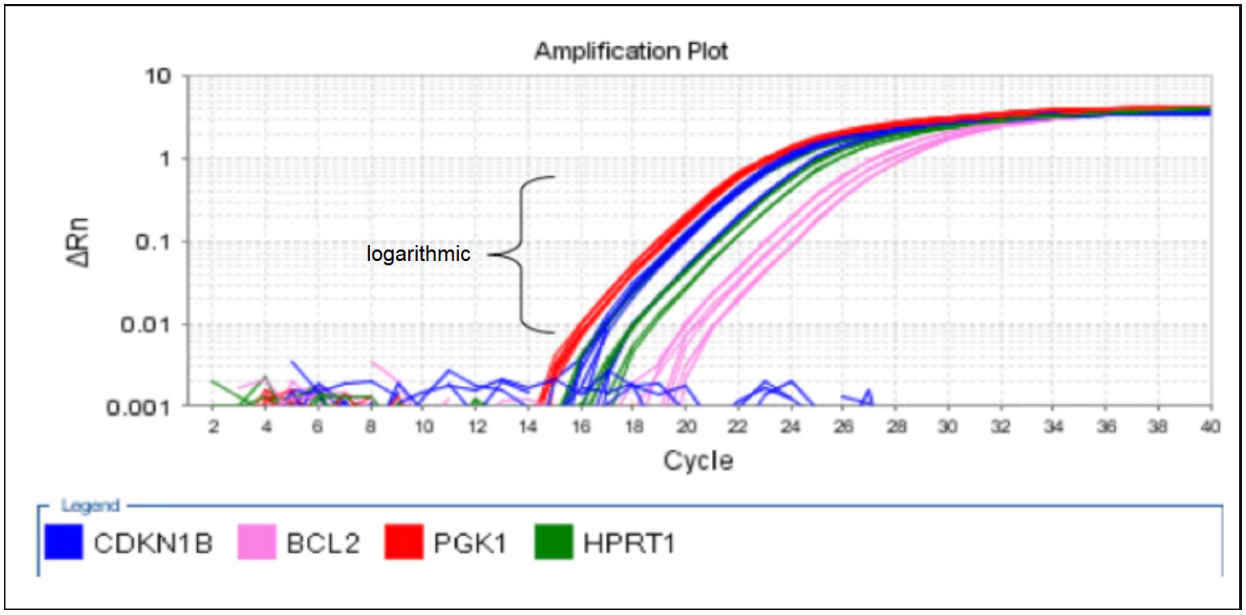


Figure 2 Visual comparison of 4 amplification plots

Verification of multiplex assay performance

Multiplex real-time PCR assay performance is the degree to which a multiplex assay can detect the gene targets that are part of the multiplex with the same accuracy and sensitivity as when the assays are run in singleplex. Multiple factors can impact multiplex assay performance, such as dye discrimination, DNA polymerase saturation and adverse primer/probe interactions. While steps can be taken to minimize the risk of these problems, wet chemistry testing is needed to verify that such preventative steps were effective.

Multiplex verification effort increases with the number of assays in the multiplex. When considering whether to create a multiplex that contains more than 2 genes per well, validation time and effort should be weighed against the long-term benefits of the multiplex.

Multiplex verification requires individual assays or oligonucleotides. Once acceptable multiplex assay performance has been verified, all the individual assays can be formulated into a single assay tube by contacting **specialty_oligos@thermofisher.com**. Use of a single-tube multiplex assay reduces labor, reduces error potential and improves primer/probe concentration consistency compared to using separate assays or oligonucleotides.

Exponential phase PCR efficiency is an issue independent of multiplexing, but the mixed standard curves used to verify multiplex performance may lead to questions about efficiency. A theoretical relationship exists between real-time PCR standard curve slopes and exponential efficiency (e): $e = 10^{-1/slope}$. However, calculating exponential efficiency using standard curve slopes is problematic, because standard curve slopes fluctuate randomly due to a variety of errors related to pipetting, mixing and other factors that occur during preparation of the standard curve. Consequently, standard curve slopes usually do not accurately reflect the true exponential phase efficiency of the assay. Care should be taken in interpreting standard curve slopes.

Multiplex verification method

The most rigorous method to verify real-time PCR multiplex assay performance is to run mixed standard curves. Mixed standard curves consist of serially diluting one gene and mixing each dilution point with a fixed amount of a second gene.

Gene amounts tested by serial dilution should correspond to that expected in real samples. The standard curve should have a minimum of 5 dilution points. If the highest and lowest gene amounts expected in samples can be estimated, the dilution factor required to span that range with at least 5 dilution points can be calculated.

The mixed dilution series is run in real-time PCR with the assays in multiplex and singleplex for comparison.

GeneArt, a part of Thermo Fisher, can synthesize plasmids or DNA strings containing the desired target sequences, which could be used for multiplex verification.

Mixed standard curve results without assay interference are illustrated in Figure 3, middle, and results with assay interference are illustrated in Figure 3, right. A deviation from the expected Ct linearity of one gene when the other gene has greater abundance is a sign that the assays are interfering with each other in the multiplex. If interference is seen, the duplex reaction can be optimized by balancing the primer and probe concentrations to an ideal state. Contact **specialty_oligos@thermofisher.com** to order separate primers and probes.

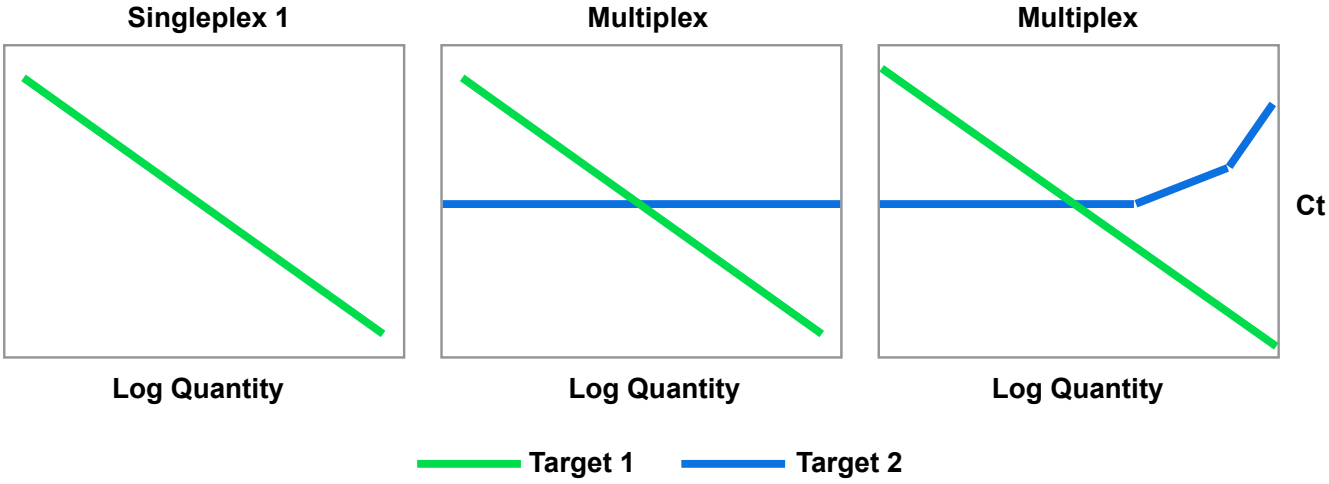


Figure 3 Illustration of possible mixed standard curve results

Mixed standard curves consist of serially diluting one gene template (Target 1) and mixing each dilution point with a fixed amount of a second gene template (Target 2).

Left: Singleplex standard curve for Target 1

Middle: Mixed standard curves for Target 1 (serial dilutions) and Target 2 (fixed amount), duplex. These curves show no interference in Target 2 Ct as Target 1 template increases.

Right: Mixed standard curves for Target 1 and Target 2, duplex, with interference. As the amount of Target 1 template increases, the Ct for Target 2 increases due to competition for reagents or other factors. Under laboratory conditions, the slope of Target 1 curve (green line) might also be distorted. When the kinetics of one reaction are more favorable than the other, the favorable reaction can be adjusted by reducing its primer concentration (primer limiting).

When verifying a triplex assay (3 assays per well), every possible duplex should be verified first before verifying the triplex. When verifying a quadruplex (4 assays per well), every possible duplex and triplex should be verified first before verifying the quadruplex assay. A mixed standard curve involving 3 genes

or more can be performed by serially diluting a mixture of two or more genes and mixing each dilution point with a fixed amount of an additional gene.

A less rigorous way to verify real-time PCR multiplex assay performance is to run a representative collection of samples in singleplex and multiplex, comparing the quantity results, relative or absolute. Using this testing procedure, the user should take additional steps to monitor multiplex assay performance. For example, amplification plots should be examined for distortions or lack of exponential PCR phase data.

Prepare mixed standard curve PCR reactions (2 targets)

Mixed standard curves consist of serially diluting one cDNA template and mixing each dilution point with a fixed amount of a second cDNA template. In this example, a dilution series of Target 1 cDNA is run, while a constant amount of Target 2 cDNA is used.

The mixed dilution series is run in real-time PCR with the assays in multiplex and singleplex for comparison.

cDNA template amounts tested by serial dilution should correspond to that expected in real samples. The standard curve should have a minimum of 5 dilution points.

1. Prepare PCR Reaction Mix in appropriately-sized microcentrifuge tubes according to the following table.

Table 19 Example PCR Reaction Mix for mixed duplex standard curves

Component	Volume per singleplex reaction (Target 1)		Volume per duplex reaction[1]	
	96–well fast (0.1–mL) plate, 384–well (0.1–mL) plate	96–well standard (0.2–mL) plate	96–well fast (0.1–mL) plate, 384–well (0.1–mL) plate	96–well standard (0.2–mL) plate
Master Mix	5 µL	10 µL	5 µL	10 µL
FAM™ dye Assay Mix (20X) (Target 1)	0.5 µL	1 µL	0.5 µL	1 µL
VIC™ dye Assay Mix (20X) (Target 2)	—	—	0.5 µL	1 µL
Nuclease–free water	1.5 µL	6 µL	1.0 µL	5 µL
Total PCR Reaction Mix volume	**7 µL**	**17 µL**	**7 µL**	**17 µL**

[1] This represents a duplex reaction. Add assay mixes for triplex or quadruplex reactions (for example, ABY™ dye orJUN™ dye), reducing the amount of Nuclease-free water accordingly.

2. Mix the components thoroughly, then centrifuge briefly to collect the contents at the bottom of the tube.

3. Transfer the appropriate volume of PCR Reaction Mix to each well of an optical reaction plate.
   - 96–well fast (0.1–mL) plate or 384–well (0.1–mL) plate: 7 µL
   - 96–well standard (0.2–mL) plate: 17 µL

4. Prepare a 10-fold dilution series of Target 1 cDNA template, with a minimum of 5 dilution points.

   Prepare a sufficient volume of the Target 1 dilution series for both the singleplex and duplex reactions.

5. Prepare a dilution of Target 2 cDNA template that results in a Ct of 27–30.

   Prepare a sufficient volume of the Target 2 for all of the duplex reactions.

6. Add up to 3 µL (total volume Target 1 plus Target 2; 1–10 ng) of cDNA template to each well.

   Add the dilution series for Target 1, and a constant amount of Target 2 template in the duplex reactions.

   If less than 3 µL of cDNA is added, add nuclease-free water to achieve a total reaction volume of 10 µL or 20 µL.

   Include a no-template control with water in place of cDNA.

7. Seal the plate with an optical adhesive cover, vortex, then centrifuge briefly to collect the contents at the bottom of each well.

8. Perform real–time PCR using the same thermal cycling conditions as for the singleplex PCR reactions (see “Set up and run the real-time PCR instrument” on page 34).

4 TaqMan™ genotyping analysis

Overview of genotyping assays

Genotyping analysis is used to perform discrimination between two alleles containing single base differences. The discrimination is based on hybridization of the probe to the allele containing the complementary sequence.

It is recommended that duplex SNP reactions be performed using one SNP genotyping assay (FAM™ dye and VIC™ dye) with the MGB–NFQ–quenched probe, and one custom SNP genotyping assay (ABY™ dye and JUN™ dye).

TaqMan™ SNP Genotyping Assays are available as predesigned assays or custom asays using the FAM™ dye and the VIC™ dye with MGB probes from the standard design pipeline. The assay with ABY™ dye and JUN™ dye must be redesigned with QSY–quenched probes.

Procedural guidelines

Primer and probe concentration

The following assay concentrations are suitable for most ABY™ dye and JUN™ dye assays.

Assay	Concentration
Forward primer	900 nM
Reverse primer	900 nM
Allele 1 probe	200 nM
Allele 2 probe	200 nM

Individual primers and probes can be ordered. A higher probe concentration can increase the signal.

Dye selection

- Pair FAM™ dye and VIC™ dye for SNP 1.
- Pair ABY™ dye and JUN™ dye for SNP 2.

Probe selection

- Use no more than two probes that contain the MGB group. This will avoid any inhibition by build–up of MGB in solution. A non-MGB probe can be designed and ordered. Contact **specialty_oligos@thermofisher.com**.
- To convert a SNP genotyping assay with FAM™ dye and VIC™ dye to a SNP genotyping assay with ABY™ dye and JUN™ dye, contact **specialty_oligos@thermofisher.com** to have assays redesigned.

IMPORTANT! The use of non–MGB probes might reduce the performance of a SNP assay due to increased heterologous probe cleavage or for other reasons. Non–MGB probes are less sensitive to single base mismatches.

Methods

Prepare singleplex PCR reactions

Ensure the performance of individual SNP reactions with ABY™ dye and JUN™ dye assays, and FAM™ dye and VIC™ dye assays, using at least one gDNA control sample for each expected genotype call and one no–template control (NTC).

1. *(Optional, if primers and probes are purchased separately.)* Prepare ABY™ dye and JUN™ dye Assay Mix, and FAM™ dye and VIC™ dye Assay Mix, according to the following table.

Concentration[1]				
Assay Mix (Final)	**Primer 1**	**Primer 2**	**Probe 1**	**Probe 2**
40X	36 µM	36 µM	8 µM	8 µM

[1] Using a 40X assay mix, the concentration of each primer in the reaction will be 900 nM and the concentration of each probe will be 200 nM.

Note: Slight changes in primer and probe concentration might be required for optimal performance.

2. Prepare the PCR Reaction Mix for each assay in an appropriately-sized microcentrifuge tube, according to one of the following tables.

Table 20 96–well fast (0.1–mL) plate and 384–well (0.1–mL) plate

Component	Volume per reaction	
	Singleplex reaction 1	Singleplex reaction 2
Master Mix	5 µL	5 µL
FAM™ dye and VIC™ dye Assay Mix (40X)	0.25 µL	—
ABY™ dye and JUN™ dye Assay Mix (40X)	—	0.25 µL
Nuclease–free water	2.25 µL	2.25 µL
Total PCR Reaction Mix volume	**7.5 µL**	**7.5 µL**

Table 21 96–well standard (0.2–mL) plate

Component	Volume per reaction	
	Singleplex reaction 1	Singleplex reaction 2
Master Mix	10 µL	10 µL
FAM™ dye and VIC™ dye Assay Mix (40X)	0.5 µL	—
ABY™ dye and JUN™ dye Assay Mix (40X)	—	0.5 µL
Nuclease–free water	6.5 µL	6.5 µL
Total PCR Reaction Mix volume	**17 µL**	**17 µL**

Note: Sample volumes can be up to 25% of the total reaction volume for crude lysates.

3. Vortex the components to mix, then centrifuge briefly to collect the contents at the bottom of each tube.

4. Transfer the appropriate volume of PCR Reaction Mix to each well of an optical reaction plate.
   - 96–well fast (0.1–mL) plate or 384–well (0.1–mL) plate: 7.5 µL
   - 96–well standard (0.2–mL) plate: 17 µL

5. Add gDNA tempate or no–template control to each well. For human assays we recommend 10–20ng DNA per PCR reaction.
   - 96–well fast (0.1–mL) plate or 384–well (0.1–mL) plate: 2.5 µL (1–10 ng of gDNA)
   - 96–well standard (0.2–mL) plate: 3 µL (2–20 ng of gDNA)

6. Seal the plate with an optical adhesive cover, vortex, then centrifuge briefly to collect the contents at the bottom of each well.

Set up and run the real-time PCR instrument

See the appropriate instrument user guide for detailed instructions to program the thermal-cycling conditions or to run the plate.

1. Select the cycling mode appropriate for the TaqPath™ ProAmp™ Multiplex Master Mix.

   IMPORTANT! The cycling mode depends on the master mix that is used in the reaction. The cycling mode does not depend on a standard or fast plate format.

2. Set up the thermal protocol for your instrument.

   Table 22 TaqPath™ ProAmp™ Multiplex Master Mix (with QuantStudio™ Real-Time PCR Systems, StepOne™ and StepOnePlus™ Real-Time PCR Instruments, ViiA™ 7 and 7500/7500 Fast Real-Time PCR Systems)

Step	Temperature	Time	Cycles
Pre-read	60°C	30 seconds	1
Initial denature / Enzyme activation	95°C	5 minutes	1
Denature	95°C	15 seconds	40
Anneal / Extend	60°C	90 seconds	
Post–read	60°C	30 seconds	1

3. Set the reaction volume appropriate for the reaction plate.
   - 96–well fast (0.1–mL) plate or 384–well (0.1–mL) plate: **10 µL**
   - 96–well standard (0.2–mL) plate: **20 µL**

4. Load the plate into the real–time PCR instrument.

5. Start the run.

6. Analyze the results.

   To ensure that the control samples were set up correctly, use one of the following tools:
   - The Genotyping application, available on Thermo Fisher™ Connect
   - The TaqMan™ Genotyper Software, available for download at **thermofisher.com/qpcrsoftware**

Prepare duplex PCR reactions

Run a duplex (4-color) SNP reaction by combining the ABY™ dye and JUN™ dye assay with the FAM™ dye and VIC™ dye assay. Use at least one gDNA control sample for each expected genotype call, and one NTC.

1. Prepare PCR Reaction Mix in appropriately-sized microcentrifuge tubes according to the following table.

Component	Volume per multiplex reaction	
	96–well fast (0.1–mL) plate and 384–well (0.1–mL) plate	96–well standard (0.2–mL) plate
Master Mix	5 µL	10 µL
FAM™ dye and VIC™ dye Assay Mix (40X)	0.25 µL	0.5 µL
ABY™ dye and JUN™ dye Assay Mix (40X)	0.25 µL	0.5 µL
Nuclease–free water	2 µL	6.0 µL
Total PCR Reaction Mix volume	**7.5 µL**	**17 µL**

2. Mix the components thoroughly, then centrifuge briefly to collect the contents at the bottom of the tube.

3. Transfer the appropriate volume of PCR Reaction Mix to each well of an optical reaction plate.
   - 96–well fast (0.1–mL) plate or 384–well (0.1–mL) plate: 7.5 µL per well
   - 96–well standard (0.2–mL) plate: 17.0 µL per well

4. Add gDNA template to each well. For human assays we recommend 10–20ng DNA per PCR reaction. For a no–template control add water in place of gDNA.
   - 96–well fast (0.1–mL) plate or 384–well (0.1–mL) plate: 2.5 µL per well
   - 96–well standard (0.2–mL) plate: 3 µL per well

5. Seal the plate with an optical adhesive cover, vortex, then centrifuge briefly to collect the contents at the bottom of each well.

6. Perform real–time PCR using the same thermal cycling conditions as the singleplex PCR reactions. See “Set up and run the real-time PCR instrument” on page 43.

Note: Do not use the StepOne™ and StepOnePlus™ systems for multiplexing more than two targets.

Analyze results

Use the Genotyping application to analyze and compare the singleplex and multiplex data. The application is available on Thermo Fisher™ Connect.

- Ensure that control sample calls are correct.
- Ensure that clusters are consistent between multiplex and singleplex and that clusters are well separated in both cases.
- Ensure that multiplex calls match those of singleplex calls.

5 TaqMan™ microRNA analysis

Overview of microRNA analysis

MicroRNA assays are used to determine the relative expression levels between different microRNA targets within a single sample. Normalization between different samples is achieved by using a reference gene. Up to two targets can be multiplexed in a single reaction depending upon the probes that are selected. In microRNA duplexing experiments, the goal is to minimize the difference between the C_t value in singleplex and duplex reactions.

Before ordering assays for duplex microRNA experiments, contact Technical Support at techsupport@thermofisher.com to request assistance with multiplex compatibility analysis. You will need to provide the two assay IDs that you wish to duplex.

Procedural guidelines

Target abundance

The amount of target and endogenous control in a sample can affect the outcome of PCR results when performing duplex assays. Abundance can be characterized by the C_t range.

Table 23 Target expression levels

Target expression level	C_t range
High	$C_t \leq 20$
Medium	$20 < C_t \leq 27$
Low	$27 < C_t \leq 35$
No–template control	$C_t > 35$

Different methods are recommended to offset the effects of target abundance when optimizing multiplex assays.

Note: This information is provided as a general guideline and might require some optimization.

Primer and probe concentrations

Optimization of the concentrations of primers and probe for each target is an important first step in assembling the duplex reaction. Guidelines for optimization will vary depending on the chemistry. For more information see the following documents:

- *TaqMan™ Advanced miRNA Assays User Guide—Single-tube Assays* (Pub. No. 100027897)
- *TaqMan™ Small RNA Assay User Guide* (Pub. No. 4364031)

Dye selection

Make dye and target assignments to balance fluorescence levels in the multiplex reaction.

- FAM™ dye can be used with low to medium expressors (typically the microRNA of interest).
- VIC™ dye can be used with medium to high expressors (typically the endogenous control).

To obtain the assay with a probe labeled with VIC™ dye email **specialty_oligos@thermofisher.com**. Include the following information:

- The assay ID of the assay you need with a probe labeled with VIC™ dye.
- The assay ID that will be duplexed with that assay. This allows us to check the sequences for multiplexing compatibility.

Duplex guidelines for TaqMan™ Advanced miRNA Assays

The PCR concentrations of the primer and probe remain unchanged from the singleplex reaction.

If the required endogenous control target is much more abundant than the miRNA of interest, request a primer-limited assay with a primer concentration of 150 nM.

Note: The goal of limiting the primer concentration in the assay is to find the primer concentration that gives the lowest (earliest) possible C_t value for the more abundant target without distorting the C_t value of the less abundant target. Limiting the primer concentration for the more abundant target should lower its ΔRn without affecting C_t.

Perform an experiment to ensure that C_t values are the same for singleplex and duplex reactions. Perform both reactions for each assay using the same dye and real–time PCR primer concentration. PCR for the singleplex and duplex reactions can be run on the same plate simultaneously.

For the duplex reaction, follow the cDNA template preparation procedure in the *TaqMan™ Advanced miRNA Assays User Guide—Single-tube Assays* (Pub. No. 100027897).

For the duplex reaction, follow the real-time PCR setup procedure in the *TaqMan™ Advanced miRNA Assays User Guide—Single-tube Assays*. Include two assays, FAM™ dye assay mix (20X) and VIC™ dye assay mix (20X), in the reaction mixture, and reduce the amount of water to account for the additional assay.

Note: The concentration for each assay in the duplex reaction should be 1X.

Duplex guidelines for TaqMan™ MicroRNA Assays

Reverse transcription (RT) requires the RT stem loop primers to be mixed together and diluted to 0.05X concentration to avoid any interaction. See the *Custom Reverse Transcription Pools and Custom Preamplification Pools with TaqMan™ MicroRNA Assays User Bulletin* (Pub. No. 4465407).

If the required endogenous control target is much more abundant then the miRNA of interest, request a primer-limited assay of forward and reverse primers at 150 nM. If a primer-limited assay is used, it must be used in both singleplex and duplex reactions.

Note: The goal of limiting the primer concentration in the assay is to find the primer concentration that gives the lowest (earliest) possible C_t value for the more abundant target without distorting the C_t value of the less abundant target. Limiting the primer concentration for the more abundant target should lower its ΔRn without affecting C_t.

Perform an experiment to ensure that C_t values are the same for the singleplex and duplex reactions. Perform both reactions for each assay. PCR for the singleplex and duplex reactions can be run on the same plate simultaneously.

For the singleplex reaction, perform RT and real–time PCR as described in the *TaqMan™ Small RNA Assay User Guide* (Pub. No. 4364031). Use the same assay (dye and real–time PCR primer concentration) in the singleplex as in the duplex reaction.

For the duplex reaction, follow the same real-time PCR setup procedure as described in the *TaqMan™ Small RNA Assay User Guide*. The following updates are required.

- Two assay mixes are added to the reaction mixture:
  - FAM™ dye assay mix
  - VIC™ dye assay mix
- The amount of water is reduced because of the additional assays.
- The final concentration of each assay in the duplex reaction is 1X.

A Supplemental information

Guidelines for primer and probe design

Guidelines for target sequence and the amplicon

A target template is a DNA sequence that will be amplified. Target templates include the following:

- cDNA
- gDNA
- plasmid DNA

Design primers to amplify amplicons (segments of DNA) within the target sequence using Primer Express™ Software or the Custom Assay Design Tool, available at **Assay Design Tool**. Consistent results can be obtained for shorter amplicons (50 to 150 bp).

Guidelines for primer design

Use Primer Express™ Software to design primers. See the *Primer Express™ Software Version 3.0 Getting Started Guide* (Pub. No. 4362460).

- The primers should be specific for the target.
  - Perform a BLAST search of the amplicon at **blast.ncbi.nlm.nih.gov/Blast.cgi**.
  - Perform *in silico* PCR using the UCSC Genome Browser at **genome.ucsc.edu/cgi-bin/hgPcr**.
- The primer melting temperature (Tm) should be the same for all primers used in the multiplex reaction.
- The length of the amplicon should be 50–150 bp for optimal PCR efficiency. If longer amplicons cannot be avoided, it may be necessary to optimize the thermal cycling protocol and reaction components.
- Ensure that primers do not contain bases that are complementary to other bases within the primer (self-complementary), or have complementarity to other primers. Complementarity at the 3' ends should be avoided to minimize the formation of artifact products, for example, primer-dimers or primer-oligomers.

  Note: AutoDimer software can be used to check primers at **https://strbase.nist.gov//AutoDimerHomepage/AutoDimerProgramHomepage.htm**.

- Do not overlap primer and probe sequences. The optimal primer length is approximately 20 bases, but may be longer or shorter depending on AT vs. GC content.
- Avoid runs of identical nucleotides. If repeats are present, there must be fewer than four consecutive G residues.
- Primers should not hybridize to regions of secondary structure within the target as these tend to have a higher melting point than the primer.

- Keep the GC content in the 40–60% range.
- Ensure that the last five nucleotides at the 3´ end contain no more than two G and/or C bases.

Guidelines for the amplicon site

Select an amplicon site that ensures amplification of the target cDNA without co-amplifying the genomic sequence, pseudogenes, and related genes. Predesigned and custom TaqMan™ Assays (when bioinformatics analysis is selected) should have high specificity for the intended targets and species. For specificity considerations related to oligonucleotide interactions, consider using the Multiple Primer Analyzer Tool (see “Primer and probe selection” on page 25.

- The amplicon should span one or more introns to avoid amplification of the target gene in gDNA.
- The primer pair must be specific to the target gene. The primer pair should not amplify pseudogenes or other related genes.
- Design primers according to the guidelines in Primer Express™ Software.
- Test the amplicons, then select those that have the highest signal-to-noise ratio. Look for low C_t with cDNA and no amplification with no template control or gDNA.
- If a sequence that adheres to the guidelines is not available, you may need to examine the sequence and redesign the amplicon or screen for more sites.
- If the gene of interest does not have introns, then an amplicon that amplifies the mRNA sequence without amplifying the gDNA sequence cannot be designed. Use RT-minus controls.

Optimize primer concentrations for real–time PCR

Overview of primer concentration

Independently vary the forward and reverse primer concentrations to identify the primer concentrations that provide optimal assay performance. Optimal primer concentrations should use the following guidelines:

- Provide a low C_t and a high ΔR_n for singleplex when run against the target template
- Provide a low C_t and a low ΔR_n when the reaction is primer–limited
- Not produce nonspecific product formation with no-template controls (NTCs)

Quantify the primers

1. Measure the absorbance of each primer oligonucleotide in TE Buffer.

   Absorbance of a 1:100 dilution is measured at 260 nm.

2. Calculate the sum of extinction coefficient contributions for each base within the primer.

   extinction coefficient contribution = Σ(extinction coefficient of a given base × number of times that base appears in the oligonucleotide sequence)

3. Calculate the oligonucleotide concentration in µM for each primer.

   Use the following equation.

absorbance at 260 nm = sum of extinction coefficient contribution × cuvette path length × concentration/100

Solve for concentration.

concentration = 100[absorbance at 260 nm / (sum of extinction coefficient contribution × cuvette path length)]

Example calculation

The concentration of a primer with the sequence CGTACTCGTTCGTGCTGC, diluted 1:100, is calculated using the following values.

Chromophore	Extinction coefficient	Number of specific chromophores in example sequence	Extinction coefficient contribution
A	15,200	1	15,200
C	7050	6	42,300
G	12,010	5	60,050
T	8400	6	50,400
Total	**—**	**—**	**167,950**

measured absorbance at 260 nm = 0.13

sum of extinction coefficient = 167,950 $M^{-1}cm^{-1}$ contributions for primer

cuvette path length = 0.3 cm

Absorbance (260 nm) = sum of extinction coefficient contributions × cuvette path length × oligonucleotide concentration/100

0.13 = 167,950 $M^{-1}cm^{-1}$ × 0.3 cm × C/100

C = 258 µM

Determine the optimal primer concentration

The objective of determining the optimal primer and probe concentrations for gene expression assays is to minimize the C_t while maintaining a ΔR_n that is reasonable for the application.

The following table provides an example of a matrix used to determine optimal primer and probe concentrations for a single template when performing gene expression analysis. The number of conditions can be reduced to fit the constraints of the block type (96-well or 384-well) and the amount

of sample and reagents. Forward and reverse primers do not necessarily have to be at the same concentration; they can be changed in certain instances, for example, primer-limiting conditions.

Condition number	Concentration		
	Forward primer	Reverse primer	Probe
1	900 nM	900 nM	250 nM
2	450 nM	450 nM	250 nM
3	300 nM	300 nM	250 nM
4	150 nM	150 nM	250 nM
5	75 nM	75 nM	250 nM
6	900 nM	900 nM	200 nM
7	450 nM	450 nM	200 nM
8	300 nM	300 nM	200 nM
9	150 nM	150 nM	200 nM
10	75 nM	75 nM	200 nM
11	900 nM	900 nM	150 nM
12	450 nM	450 nM	150 nM
13	300 nM	300 nM	150 nM
14	150 nM	150 nM	150 nM
15	75 nM	75 nM	150 nM
16	900 nM	900 nM	100 nM
17	450 nM	450 nM	100 nM
18	300 nM	300 nM	100 nM
19	150 nM	150 nM	100 nM
20	75 nM	75 nM	100 nM

1. Prepare a reaction plate using the desired templates and single template concentrations.
2. Determine the concentration combinations to be evaluated and prepare the reactions accordingly.
3. Prepare the reaction plate.
4. Set up and run the real–time PCR instrument.
5. Analyze the results, and compile ΔR_n and C_t values to select the concentrations that give high ΔR_n values and low C_t values.

Note: For SNP assay formulation, we recommend starting with the concentration of predesigned assays (900 nM for each primer and 200 nM for each probe).

Best practices for PCR and RT-PCR experiments

Good laboratory practices for PCR and RT-PCR

- Wear clean gloves and a clean lab coat.
  - Do not wear the same gloves and lab coat that you have previously used when handling amplified products or preparing samples.
- Change gloves if you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation and reaction setup.
  - Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or DNA decontamination solution.

Use UNG to prevent false-positive amplification

Carryover amplicons can result in false-positive amplification during PCR. Use a master mix that contains heat-labile uracil-N-glycosylase (UNG; also known as uracil-DNA glycosylase (UDG)) to degrade many contaminating carryover amplicons.

UNG enzymatic activity occurs during the PCR reaction setup at room temperature; an activation step before thermal cycling is not necessary. Unlike standard UNG, heat-labile UNG is completely inactivated during the first ramp to the high-temperature step for template denaturation and polymerase activation.

To ensure the desired UNG activity:

- Use PCR components and thermal cycling conditions as specified.

  UNG-containing master mixes incorporate the optimal concentration of UNG to prevent cross-contamination while not affecting real-time PCR performance.
- Do not attempt to use UNG-containing master mixes in subsequent amplification of dU-containing PCR products, such as in nested-PCR protocols. The UNG will degrade the dU-containing PCR products, preventing further amplification.

Although treatment with UNG can degrade or eliminate large numbers of carryover PCR products, use good laboratory practices to minimize cross-contamination from non-dU-containing PCR products or other samples.

Detect fluorescent contaminants

Fluorescent contaminants can generate false positive results. To help detect these contaminants, we recommend including a no-amplification control reaction that contains sample, but no master mix.

After PCR, if the absolute fluorescence of the no-amplification control is greater than the fluorescence of the no template control (NTC), fluorescent contaminants may be present in the sample or in the heat block of the real-time PCR instrument.

B

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:
  www.who.int/publications/i/item/9789240011311

C Documentation and support

Related documentation and software

Document	Pub. No.
TaqMan™ SNP Genotyping Assays User Guide	MAN0009593
TaqMan™ Advanced miRNA Assays User Guide—Single-tube Assays	100027897
TaqMan™ Gene Expression Assays User Guide—single-tube assays	4333458
Introduction to Gene Expression Getting Started Guide	4454239
TaqMan™ Small RNA Assay User Guide	4364031
Custom Reverse Transcription Pools and Custom Preamplification Pools with TaqMan™ MicroRNA Assays User Bulletin	4465407
Master Mixes	**Pub. No.**
TaqMan™ Multiplex Master Mix Quick Reference	MAN0009605
TaqPath™ ProAmp™ Master Mixes User Guide	MAN0015758
TaqPath™ 1-Step Multiplex Master Mix User Guide	MAN0014269
Software	**SKU**
Primer Express™ Software Version 3.0 Getting Started Guide	4362460
Custom Assay Design Tool	Available at **Assay Design Tool**
TaqMan™ Genotyper Software User Guide	4448637

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- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

ThermoFisher SCIENTIFIC

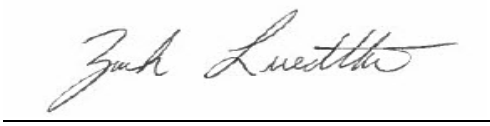
Life Technologies CERTIFICATE OF ANALYSIS

Catalog Number Q32851
Product Name Qubit™ dsDNA HS Assay Kit, 100 assays
Lot Number 2663870

	LOT DATA	SPECIFICATION
ABSORPTION[1] OD ± 0.05 at lambda max ± 5 nm	meets specification	OD ± 0.05 at lambda max ± 5 nm
ABSORPTION Optical Density of Component D[2]	0.194 at 260 nm	0.2 ± 0.008 at 260 nm

1. Solvent: 50 mM potassium phosphate buffer, pH 7. Method: Optical Density: 20X dilution.

2. Solvent: Buffer, TE Buffer. Method: Optical Density. undiluted



Zach Luedtke, Quality Assurance Manager
14-Jun-2023

Life Technologies Corporation certifies on the date above that this is an accurate record of the analysis of the subject lot, and that the data conform to the specifications in effect for this product at the time of analysis.

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Printed Jun 15, 2023

ThermoFisher SCIENTIFIC

Nucleic acid quantification

Qubit fluorometers and assays

Accurate, specific, and sensitive quantification of DNA, RNA, and protein samples

invitrogen

Qubit fluorometers

Intuitive user interface coupled with accurate measurements

Invitrogen™ Qubit™ 4 and Qubit™ Flex Fluorometers are benchtop microvolume fluorometers designed to accurately measure DNA, RNA, or protein quantity. Whether you are an expert or a novice, the easy-to-use touchscreen menus make it easy to perform assays, with accurate and reliable results displayed in just a few seconds. Both instruments provide flexible data exportation using a USB drive, Wi-Fi cloud connectivity, or direct USB cable connection so your quantification data are easily accessed.

Key benefits of Qubit fluorometers

- **High sensitivity**—more sensitive than UV absorbance–based quantification
- **Accuracy and speed**—accurately quantifies DNA, RNA, or protein in less than 3 seconds
- **Ideal for precious samples**—requires as little as 1 µL of sample
- **Optimized reagents and tubes**—Invitrogen™ Qubit™ reagents and assay tubes work best with Qubit fluorometers

Figure 1. Qubit Flex and Qubit 4 Fluorometers with intuitive touchscreens and applications for specific assays.

Qubit fluorescence technology

Qubit fluorometers and assay kits are designed to measure the intensity of the signal from fluorescent dyes bound to specific biological molecules. These optimized dyes bind selectively to DNA, RNA, or protein and only emit a fluorescent signal when bound to the target.

Qubit fluorometers use specialized curve-fitting algorithms to develop a calibration curve using standard samples with a known concentration. An unknown sample concentration of DNA, RNA, or protein is calculated by comparing the relative fluorescence units (RFUs) of the sample to the RFUs of the standards used in calibration. The detection limits of the measurements are specific to each assay.

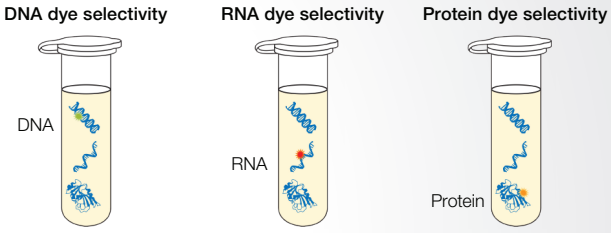


Figure 2. Fluorescent dyes selectively bind to DNA, RNA, or protein. Dyes only emit signal when bound to the target.

Convenient, easy-to-use onboard calculators

Reagent calculator

The reagent calculator conveniently calculates how much working solution to prepare based on the number of samples to quantify. Available in both the Qubit 4 and Qubit Flex Fluorometer models.

Assay range calculator

The assay range calculator displays the core sample concentration range based on the sample volume, as well as the extended low and high ranges. This aids in the selection of the appropriate Qubit assay for the most accurate quantification based on your sample volume and estimated sample concentration. This calculator is only available with the Qubit Flex Fluorometer.

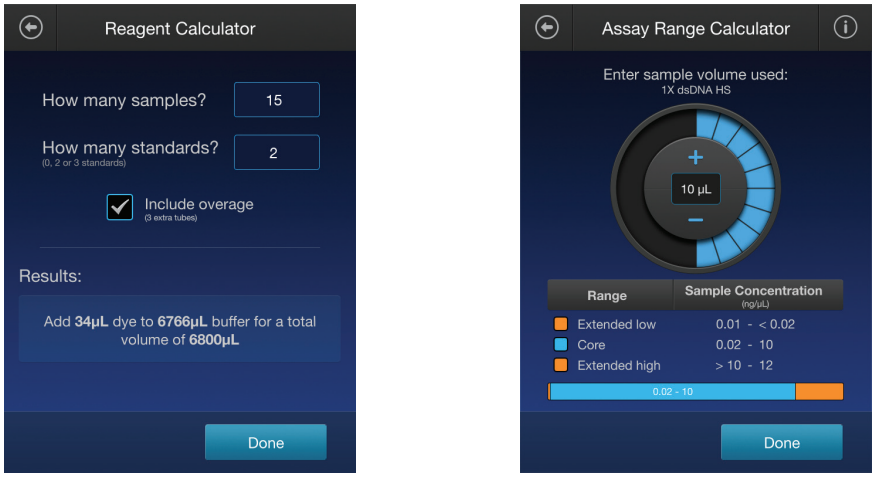


Figure 3. Reagent and assay range calculators. Easily make working solutions for all assays that are not in 1X format using the reagent calculator. The assay range calculator aids in the determination of sample volume requirements based on required accuracy.

Calculators for next-generation sequencing (NGS) workflows

Molarity calculator

Quickly calculate the molarity of your samples based on nucleic acid length and the measured concentration. The molarity calculator is only available on the Qubit Flex Fluorometer.

Normalization calculator

Easily normalize to a desired mass, concentration, or molarity with the normalization calculator. This replaces spreadsheet calculations for standard normalization during library preparation for sequencing. The normalization calculator is only available on the Qubit Flex Fluorometer.

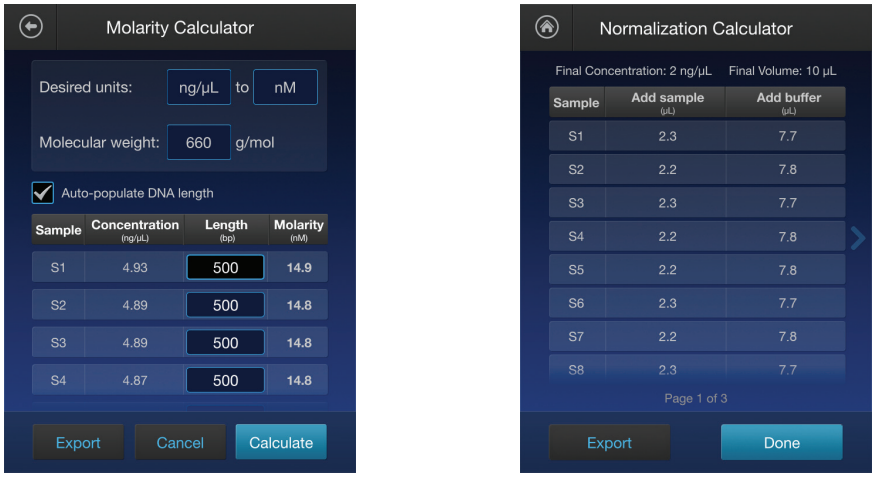


Figure 4. Integrated post-results molarity and normalization calculators. Use the molarity calculator to convert values to molarity based on nucleic acid length. Use the normalization calculator to determine how to dilute the samples to the same concentration.

Personalized workflows

Envision and create custom assays for the Qubit 4 Fluorometer

MyQubit functionality brings your favorite fluorescence assays right to your benchtop, providing a reliable platform for many quantitation needs—from laboratory research and quality control to process monitoring and beyond. Any fluorescent reagent or assay that is spectrally compatible with the Qubit hardware can be adapted for use with the Qubit 4 Fluorometer.

Compare Qubit fluorometers

	Qubit 4 Fluorometer	Qubit Flex Fluorometer
Sample throughput	1 sample in 3 seconds	1 to 8 samples in 3 seconds
User interface	5.7 in. color touchscreen	8 in. color touchscreen
Onboard calculators	Reagent calculator	Reagent calculator Assay range calculator Molarity calculator Normalization calculator
Informs where the sample concentration resides within the assay range	Provides quantification data for samples that are within the core and the extended range of the standard curve. Sample concentrations that are out of range are not given a measurement.	
System check	Qubit 4 System Verification Assay Kit	Qubit Flex System Verification Assay Kit
Fluorometer mode	Yes	No
Programable open format	Yes—MyQubit	No
Instrument footprint (W x L x H)	13.6 x 25 x 5.5 cm 5.4 x 10 x 2.2 in.	1.86 x 28.2 x 10.3 cm 7.3 x 11.1 x 4.1 in.
Sample data storage	1,000 samples	10,000 samples
Data export	Wi-Fi USB drive Direct to computer via USB or ethernet cable	Wi-Fi USB drive Direct to computer via USB or ethernet cable
Light sources	Blue LED (peak ~470 nm), Red LED (peak ~635 nm)	Blue LED (peak ~460–480 nm), Red LED (peak ~620–640 nm)
Excitation filters	Blue LED (430–495 nm), Red LED (600–645 nm)	Blue LED (456–484 nm), Red LED (612–644 nm)
Emission filters	Green (510–580 nm), Red (665–720 nm)	Green (513–563 nm), Far-red (671–693 nm)

21 CFR Part 11 compliance support software

The Qubit Flex Fluorometer combines high-performance nucleic acid and protein quantifications with regulatory compliance support for busy labs that need efficient processes. Benefits include:

- **High performance**—get accurate and reproducible quantification in seconds
- **Security, audit, and e-signature (SAE) software**—for 21 CFR Part 11 compliance support of electronic record keeping
- **Intuitive interface**—simple to use, with several convenient calculators
- **Multiple assays**—Invitrogen™ Qubit™ DNA, RNA, and protein quality and integrity assays are available

Learn more about Qubit fluorometers with SAE software for 21 CFR Part 11 compliance support.

Qubit 4 and Qubit Flex kits for system verification

The Invitrogen™ Qubit™ 4 System Verification Assay Kit and the Invitrogen™ Qubit™ Flex System Verification Assay Kit are fast, easy-to-use, reagent-based assays that test the performance of Qubit fluorometers. Each kit consists of three components: a blank reagent solution, a green fluorescent reagent, and a far-red fluorescent reagent. Paired with a hardware functionality test, the assay is designed to test the internal components of the instrument to help ensure proper functionality.

Qubit RNA quantification assays

There are three RNA assay kits, which offer differing detection ranges, and one microRNA assay kit:

- **Invitrogen™ Qubit™ RNA HS Assay Kit**—high sensitivity
- **Invitrogen™ Qubit™ RNA BR Assay Kit**—broad range
- **Invitrogen™ Qubit™ RNA XR Assay Kit**—extended range
- **Invitrogen™ Qubit™ microRNA Assay Kit**—highly selective for miRNA over rRNA or large mRNA (>1,000 bp)

The RNA assays are accurate for initial sample concentrations from as little as 250 pg/µL to 10,000 ng/µL. These kits are highly selective for RNA over dsDNA. Unlike other RNA assays, they do not require DNase if DNA is present in the sample for an accurate measurement.

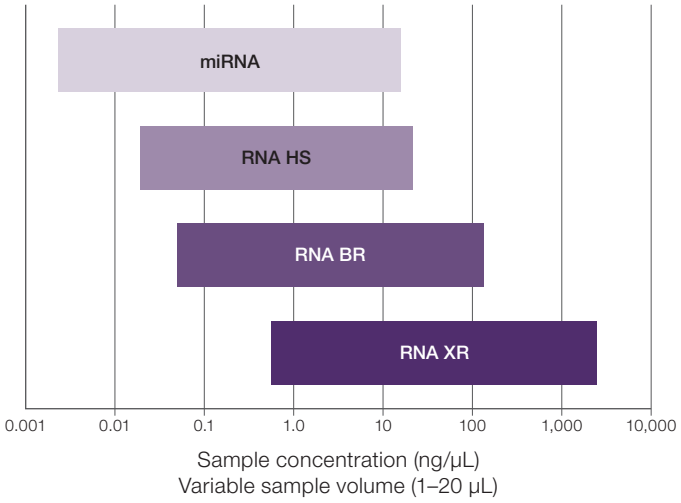


Figure 5. Quantification ranges of Qubit RNA assay kits.

Qubit RNA integrity and quality (IQ) assay

The Invitrogen™ Qubit™ RNA IQ Assay was developed to quickly assess the quality and integrity of an RNA sample. This assay allows assessment of RNA quality at a lower cost and with an easy-to-use, intuitive workflow.

The Qubit RNA IQ Assay utilizes two unique dyes—one that binds to large, intact, and/or structured RNA, and another that selectively binds to small, degraded RNA. Together, they are able to quickly assess the quality and integrity of an RNA sample. To use, simply add your samples to the Qubit RNA IQ working solution, then measure on the Qubit 4 or Qubit Flex Fluorometer.

Results are presented as a total value for the RNA sample integrity and quality, or RNA IQ number, and as the calculated percentage of large and small RNA in the sample. The RNA IQ number is based on a scale of 1 to 10, wherein a high IQ number indicates the majority of the sample consists of large and/or structured RNA. Conversely, a small IQ number indicates the sample comprises mainly small RNA with limited tertiary structure.

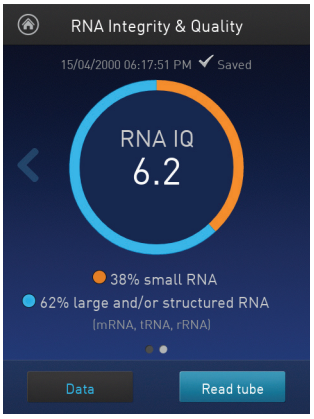


Figure 6. A proprietary algorithm is used to report a quality score representative of the ratio of small and large and/or structured RNA in the sample.

Qubit DNA quantification assays

Invitrogen™ Qubit™ DNA assay kits are broadly categorized as double-stranded DNA (dsDNA) assays or single-stranded DNA (ssDNA) assays.

Qubit dsDNA assay kits—available in two detection ranges and two formats

Detection ranges: high-sensitivity and broad-range assays

- The Invitrogen™ Qubit™ dsDNA High-Sensitivity (HS) Assay Kit is for samples with a low concentration of dsDNA, making it ideal for precious samples; it has a detection range between 0.1 and 120 ng
- The Invitrogen™ Qubit™ dsDNA Broad-Range (BR) Assay Kit is ideal for a broad range of DNA concentrations and applications; it can detect between 4 and 4,000 ng

Use high-sensitivity (HS) assays for low concentrations and broad-range (BR) assays for high concentrations of dsDNA

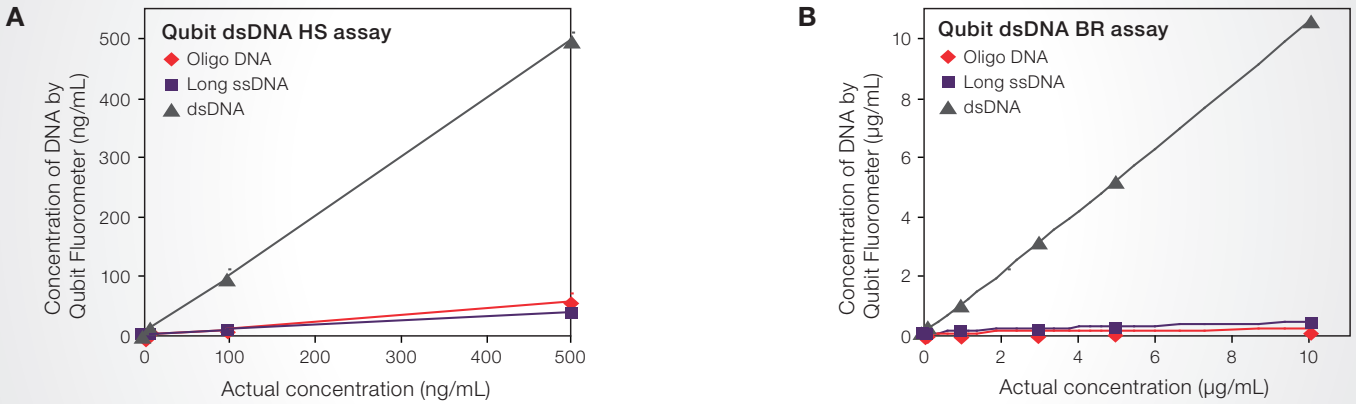


Figure 7. Detection of double-stranded DNA by the Qubit dsDNA HS (A) and BR (B) assay kits. Duplicate samples of long ssDNA, oligo DNA, or lambda dsDNA at concentrations of 0.5 to 500 ng/mL in the assay tube were quantified using the Qubit dsDNA HS assay, and at concentrations of 0.01 to 10 µg/mL in the assay tube using the Qubit dsDNA BR assay according to kit protocols.

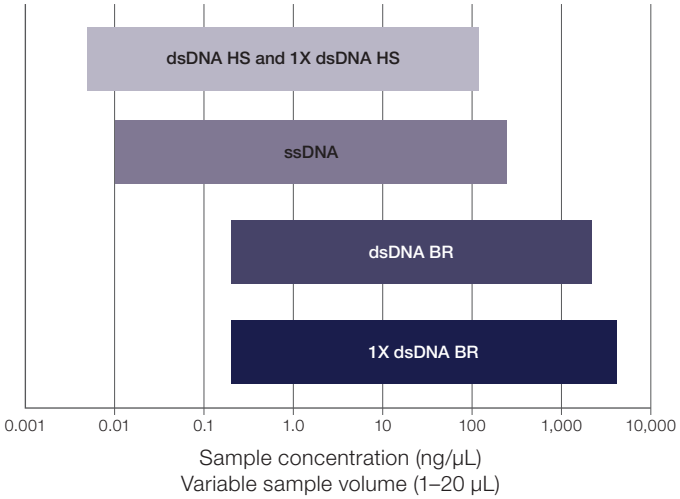


Figure 8. Quantification ranges of Qubit DNA assay kits.

Formats: standard assay and 1X assay

- Invitrogen™ Qubit™ standard assays require same-day mixing of the buffer with the reagent to create the working solution prior to preparing standards and samples for quantification
- Invitrogen™ Qubit™ 1X assays eliminate the step of preparing the working solution
  - The Invitrogen™ Qubit™ 1X dsDNA HS Assay Kit provides the same dynamic range and limit of detection as the standard assay, while the Invitrogen™ Qubit™ 1X dsDNA BR Assay Kit has a wider dynamic range than the standard assay, achieving 4,000 ng/µL in the extended range
  - This format offers a simplified workflow while reducing the tubes in the kit, therefore reducing the amount of plastic used
  - Simply add your sample or standard to the premixed solution, incubate, and read your results

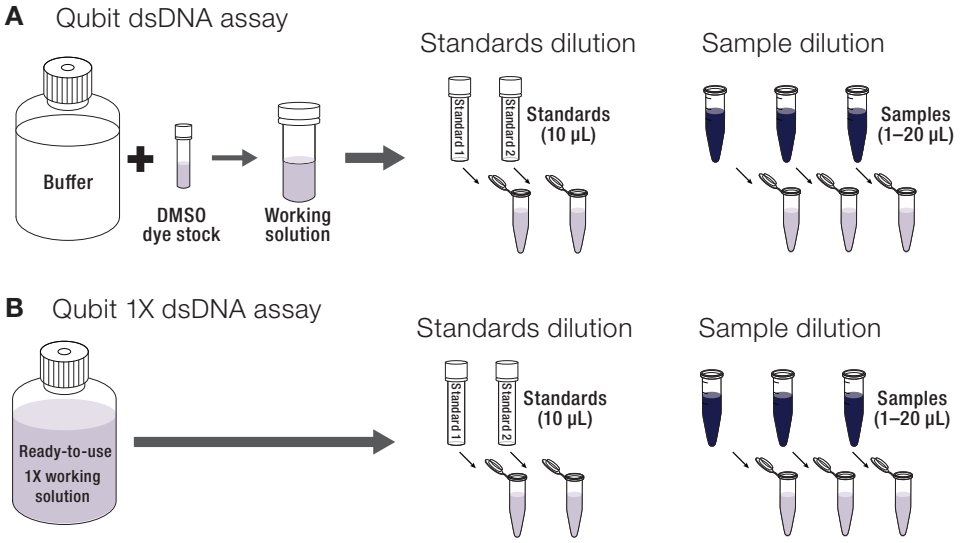


Figure 9. Workflow comparison for the (A) Qubit dsDNA and (B) Qubit 1X dsDNA assays. Standard Qubit dsDNA High Sensitivity (HS) and Qubit dsDNA Broad Range (BR) assay kits include a fluorogenic dye, buffer, and dsDNA standards. Prior to each assay, a fresh aqueous working solution needs to be prepared by diluting the dye stock in the provided buffer in a 1:200 ratio. Qubit 1X dsDNA assay kits eliminate this step by providing a ready-to-use working solution.

Qubit ssDNA and oligos quantification assays

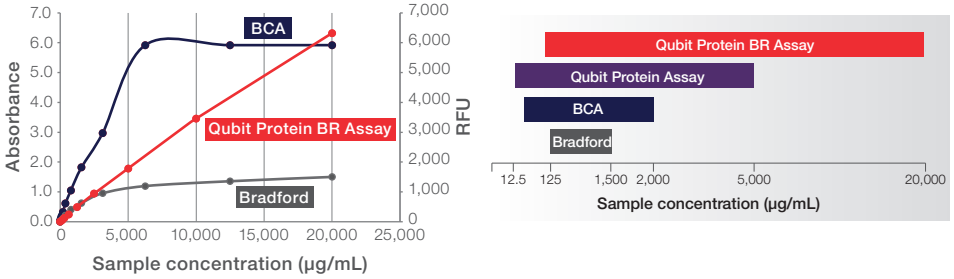
The Invitrogen™ Qubit™ ssDNA Assay Kit is ideal for quantifying single-stranded DNA or oligonucleotides. It is accurate for initial sample concentrations from 50 pg/µL to 200 ng/µL, providing an assay range of 1–200 ng.

Qubit assays

Qubit assays are designed to work with Qubit fluorometers. Common contaminants such as salts, free nucleotides, RNA, solvents, detergents, and proteins are well tolerated in Qubit assays.

Qubit protein quantification assays

Invitrogen™ Qubit™ protein assay kits are designed to make protein quantification easy and fast. The assays provide low protein variability, rapid quantitation, and high sensitivity. Common contaminants, such as reducing reagents (DTT, β-mercaptoethanol), salts, free nucleotides, amino acids, solvents, DNA, and detergents (Invitrogen™ Qubit™ Protein BR Assay only), are well tolerated in the assays. The assays' wide dynamic ranges make it easy to determine the concentrations of a wide range of samples compared to standard colorimetric protein assays.



	Qubit Protein BR Assay	Qubit Protein Assay
Platform	Qubit 4 Fluorometer	Qubit 4 and Qubit Flex Fluorometers
Compatibility	Detergents, reducing agents	Reducing agents
Quantitation range	100 µg/mL to 20 mg/mL	12.5 µg/mL to 5 mg/mL

Figure 10. Quantitation ranges of protein assays.

Qubit endotoxin detection assay

The Invitrogen™ Qubit™ Endotoxin Detection Assay Kit is a sensitive and easy-to-use fluorescent endpoint assay that uses amebocyte lysates to quantify endotoxin in protein, peptides, antibodies, or nucleic acid samples. Amebocyte lysates are widely used as a simple and sensitive assay for the detection of endotoxin lipopolysaccharide (LPS), the membrane component of gram-negative bacteria.

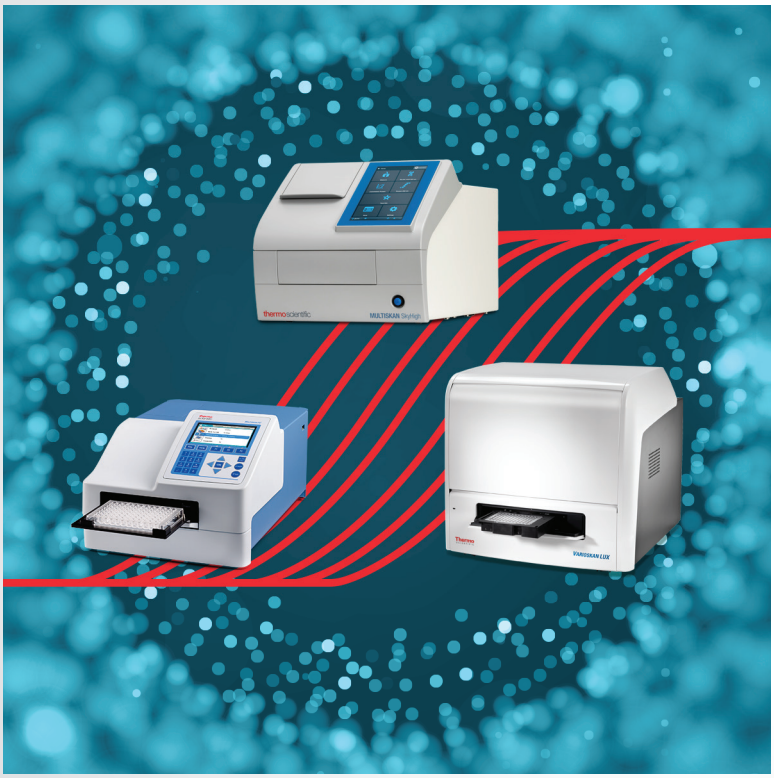
When endotoxin encounters the amebocyte lysate, a series of enzymatic reactions result in the activation of Factor C, Factor B, and pro-clotting enzyme. The activated enzyme catalyzes a cleavage event in the substrate to produce a strong fluorescent signal. After stopping the reaction, the resulting signal is measured on the Qubit Flex Fluorometer. This fluorescent signal is proportional to the endotoxin concentration in the sample.

Key features and benefits of the Qubit Endotoxin Detection Assay Kit include:

- **Highly sensitive with a broad range**—detect as little as 0.01–10.0 EU/mL
- **Suitable for a wide range of samples**—including proteins, plasmid preparations, DNA, and RNA
- **Easy to use**—when paired with the Qubit Flex Fluorometer, calculations are performed automatically, reducing the potential for error

Determining endotoxin levels is important to assess the efficiency of endotoxin removal methods and prevent endotoxic shock, inflammation, and/or sepsis in tissue culture cells and animals injected with endotoxin-contaminated proteins.

Need higher throughput for your nucleic acid or protein samples?



Qubit assays are ideal when the number of samples you measure at one time is low enough not to warrant a microplate reader. With larger sample batches requiring a fluorescence microplate reader, use Invitrogen™ Quant-iT™ assay kits and reagents, which are designed to use with microplate readers for nucleic acid or protein quantification.

Learn more about Quant-iT assays at
thermofisher.com/quantit

Learn more about microplate readers at
thermofisher.com/platereaders

Resources

Explore online and download materials for Qubit fluorometric quantitation including manuals, guides, brochures, information sheets, interactive 3D virtual tours, demos, videos, citations, articles, posters, technical documentation, and application notes.

thermofisher.com/qubitresources

Frequently asked questions

Q. I already have a Thermo Scientific™ NanoDrop™ instrument; why should I use a Qubit fluorometer?

A. NanoDrop instruments use UV absorbance to measure DNA and RNA concentrations. Absorbance-based measurements have limitations in distinguishing between DNA, RNA, and free nucleotides, which absorb at 260 nm.

Qubit assays are fluorescence-based. They are designed to only quantify the target analyte. Additionally, fluorescence-based nucleic acid quantification provides a more sensitive dynamic range than absorbance-based instruments.

When used with Qubit assays, Qubit fluorometers can accurately measure low concentrations of sample, while NanoDrop spectrophotometers can detect the presence of common contaminants.

Q. Do I have to use new standards every time?

A. For each assay, you have the choice to run a new calibration or to use the values from the previous calibration. As you first use the instrument, perform a new calibration each time. As you become familiar with the assays, the instrument, your pipetting accuracy, and significant temperature fluctuations within your laboratory, you can determine the level of comfort you have using the calibration data stored from the last time the instrument was calibrated. We do recommend running a new calibration curve every time you prepare a new working solution.

Q. Is there a difference in signal between supercoiled and relaxed plasmid DNA when using a Qubit fluorometer?

A. Yes, we have seen a 20–30% difference. For the different forms of plasmid DNA, we recommend using a standard that more closely represents the composition of the plasmid DNA in the sample.

Q. Does the Qubit Protein Assay or Qubit Protein BR Assay work well in the presence of detergents?

A. The Qubit Protein BR Assay is compatible with samples that contain up to 5% detergents. The Qubit Protein Assay is not recommended if detergent is present.

Q. Why are some of the instrument setting menu options not available on my Qubit 4 or Qubit Flex device?

A. To adhere to cybersecurity legal standards, the latest firmware for Qubit 4 and Qubit Flex Fluorometers will require users to log in to access certain menu options (such as instrument settings, software update, and system verification). When not logged in to a user profile, these menu options will appear to be inactive.

Q. Is there a way to verify that my Qubit 4 or Qubit Flex Fluorometer is functioning properly?

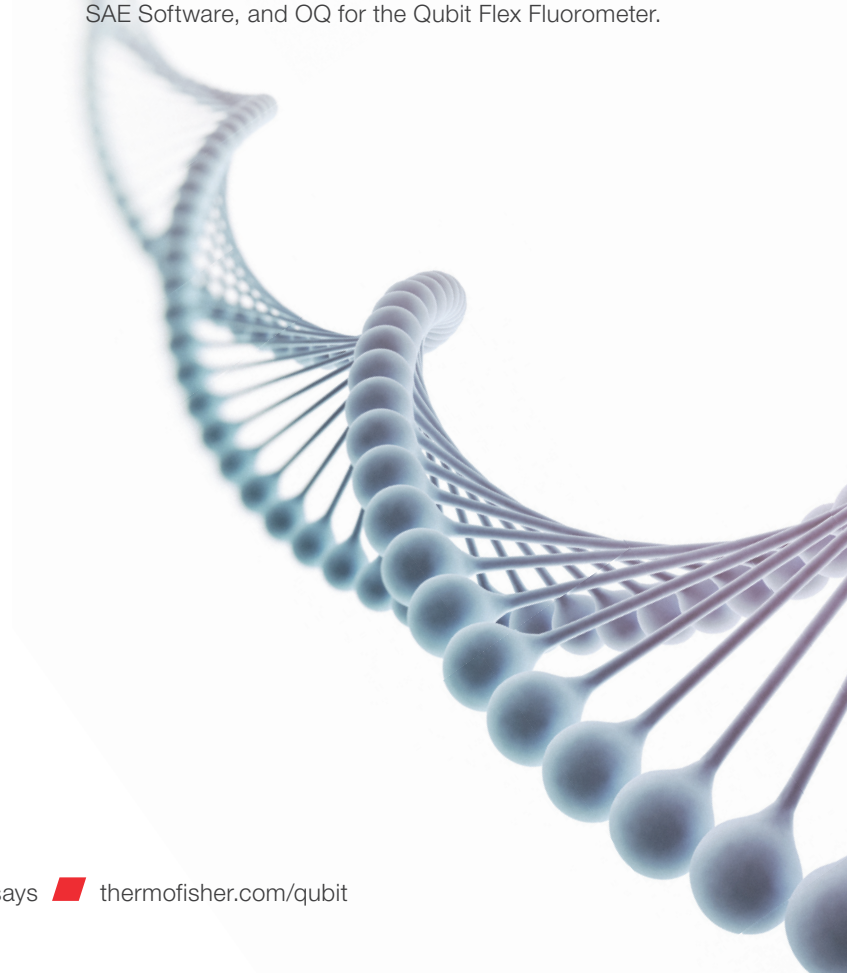
A. Qubit 4 and Qubit Flex System Verification Assay Kits offer a fast, easy-to-use, reagent-based method to test the internal components of a Qubit fluorometer. Perform the system verification when a problem with the instrument is suspected. It is not necessary to perform verification regularly.

Q. How long does the lamp last?

A. There are two LED light sources in the Qubit 4 and Qubit Flex Fluorometers. They are expected to last 5 years.

Q. Do Qubit fluorometers offer qualification services?

A. Yes, our manufacturer-trained and -certified field service engineers (FSEs) can perform installation qualification (IQ) and operational qualification (OQ). We offer options including IQ/OQ for the Qubit Flex Fluorometer, IQ/OQ for Qubit Flex SAE Software, and OQ for the Qubit Flex Fluorometer.



Ordering information

Instruments and accessories	Quantity	Cat. No.
Qubit 4 Fluorometer		
Qubit 4 Fluorometer (w/ Wi-Fi)	1 instrument	Q33238
Qubit 4 Quantitation Starter Kit (w/ Wi-Fi)	1 kit	Q33239
Qubit 4 NGS Starter Kit (w/ Wi-Fi)	1 kit	Q33240
Qubit 4 RNA IQ Starter Kit (w/ Wi-Fi)	1 kit	Q33241
Qubit 4 Protein BR Assay Starter Kit (w/ Wi-Fi)	1 kit	A51292
Qubit Assay Tubes	500 tubes	Q32856
Qubit 4 System Verification Assay Kit	50 assays	Q33237
Qubit Flex Fluorometer		
Qubit Flex Fluorometer	1 instrument	Q33327
Qubit Flex NGS Starter Kit	1 kit	Q45893
Qubit Flex Quantitation Starter Kit	1 kit	Q45894
Qubit Flex Assay Tube Strips	125 tube strips	Q33252
Qubit Flex Assay Reservoirs	100 reservoirs	Q33253
Qubit Flex System Verification Assay Kit	25 assays	Q33254
Qubit Flex Fluorometer with SAE Software for 21 CFR Part 11 Support	1 package	Q45895
Qubit Flex SAE Software for 21 CFR Part 11 Support	1 license	Q31994

Product	Initial sample concentration	Quantitation range	Quantity	Cat. No.
DNA quantification assays				
dsDNA HS assays				
Qubit 1X dsDNA HS Assay Kit	0.005–120 ng/µL	0.1–120 ng	100	Q33230
			500	Q33231
Qubit dsDNA HS Assay Kit	0.005–120 ng/µL	0.1–120 ng	100	Q32851
			500	Q32854
Qubit 1X dsDNA HS Assay Lambda Standard	-	-	-	Q33233
dsDNA BR assays				
Qubit 1X dsDNA BR Assay Kit	0.2–4,000 ng/µL	4–4,000 ng	100	Q33265
			500	Q33266
Qubit dsDNA BR Assay Kit	0.2–2,000 ng/µL	4–2,000 ng	100	Q32850
			500	Q32853
Qubit 1X dsDNA BR Assay Lambda Standards	-	-	-	Q33263
ssDNA and oligos assay				
Qubit ssDNA Assay Kit	0.05–0.2 ng/µL	1–200 ng	100	Q10212
RNA quantification assays				
Qubit RNA HS Assay Kit	250 pg/µL and 100 ng/µL	5–100 ng	100	Q32852
			500	Q32855
Qubit RNA BR Assay Kit	1 ng/µL to 1 µg/µL	20–1,000 ng	100	Q10210
			500	Q10211
Qubit RNA XR Assay Kit	10 ng/µL and 10,000 ng/µL	200–10,000 ng	100	Q33223
			500	Q33224
Qubit microRNA Assay Kit	50 ng/mL to 100 µg/mL	1–1,000 ng	100	Q32880
			500	Q32881

Ordering information (continued)

Product	Quantity	Cat. No.
RNA IQ assays		
Qubit RNA IQ Assay Kit	75 assays	Q33221
	275 assays	Q33222
Qubit RNA IQ Standards	1 set	Q33235

Product	Fluorometer	Initial sample concentration	Quantity	Cat. No.
Protein assays				
Qubit Protein Assay Kit	Qubit Flex, Qubit 4	12.5 µg/mL to 5 mg/mL	100	Q33211
			500	Q33212
Qubit Protein BR Assay Kit	Qubit 4	100 µg/mL to 20 mg/mL	100	A50668
			500	A50669

Product	Fluorometer	Quantity	Cat. No.
Endotoxin assays			
Qubit Endotoxin Detection Assay	Qubit Flex	80 assays	Q32891
Qubit Flex Pyrogen-Free Tube Strips	Qubit Flex	120 tube strips	Q32893
Qubit Flex Endotoxin Starter Kit	Qubit Flex	1 kit	Q32894

Learn more at **thermofisher.com/qubit**

invitrogen

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Using ExoSAP-IT™ *Express* PCR Product Cleanup to generate high quality BigDye™ Terminator v3.1 Cycle Sequencing Kit Data

Publication Number MAN0017063 **Revision** A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Overview

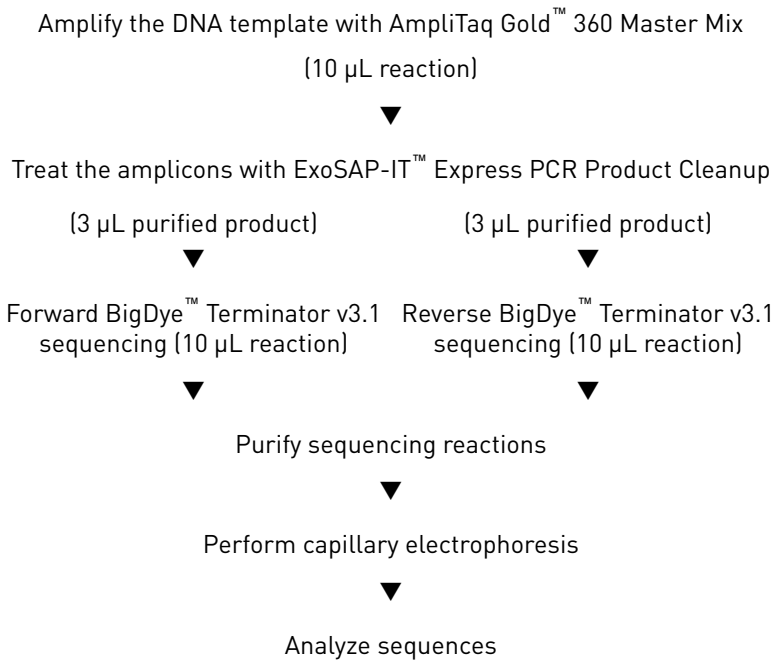
This document provides a protocol for generating high-quality sequence data using:

- BigDye™ Terminator v3.1 Cycle Sequencing Kit
- Applied Biosystems™ Genetic Analyzers
- ExoSAP-IT™ *Express* PCR Product Cleanup

Certain components of the protocol workflow such as reagent kits and other protocols for preparation of reagents may not be available through Thermo Fisher Scientific.

For sequencing short amplicons, use the alternative protocol, *Generating high-quality data using the BigDye™ Direct Cycle Sequencing Kit* (Pub. no. MAN0014436), which has been optimized to reduce loss of 5′ sequences.

Workflow



Required materials

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Item	Source
Reagents	
BigDye™ Terminator v3.1 Cycle Sequencing Kit	4337456
AmpliTaq Gold™ 360 Master Mix	4398881
BigDye XTerminator™ Purification Kit	4376486
UltraPure™ DNase/RNase-Free Distilled Water	10977-015
Hi-Di™ Formamide **Note:** Not required for BigDye XTerminator™ Purification Kit purification.	4311320 or 4440753
ExoSAP-IT™ *Express* PCR Product Cleanup	75001
DNA Suspension Buffer, RNAse DNAse Free (10 mM Tris/0.1 mM EDTA, pH 8.0)	Teknova, Inc. T0223
PCR and sequencing primers (HPLC-purified recommended)	Primers can be designed, chosen, and ordered with the Primer Designer™ Tool at **http://www.thermofisher.com/primerdesigner**
Reagents for Centri-Sep™ purification (*optional*)	

Item	Source
Sodium Dodecyl Sulfate (SDS)	15525-017
Centri-Sep™ 96-Well Plates	4367819
Reagents for ethanol/EDTA purification (*optional*)	
0.5M EDTA, pH 8.0 for molecular biology	AM9260G
Ethanol, absolute, for molecular biology	Major Laboratory Suppliers (MLS)
Laboratory supplies	
MicroAmp™ Clear Adhesive Film	4306311
MicroAmp™ Optical 96-Well Reaction Plate	8010560
Plate Septa, 96 well	4315933
25 mL Reagent Reservoir, Pyrogen-free, RNase/DNase certified, sterile	VistaLab Technologies, Inc. 3054-1002
Digital Vortex-Genie™ 2 or equivalent	Scientific Inducstries, Inc. SI-A536
Centrifuge with swinging bucket (with PCR plate adapter)	MLS
Compatible thermal cyclers [1]	
GeneAmp™ PCR System 9700	Contact your local sales office
Veriti™ Thermal Cycler	
Compatible genetic analyzers	
3130/3130*xl* Genetic Analyzer	Contact your local sales office
3500/3500xL Genetic Analyzer	
3730/3730*xl* DNA Analyzer [2]	

[1] If you use a different thermal cycler, you may need to optimize the thermal cycling conditions.

[2] Standard heat seal consumables can be used in place of MicroAmp™ plates and film for these instruments.

DNA and primer requirements

Input DNA requirements

The quality of the DNA can significantly influence the length of the fragment that can be amplified and the reproducibility of amplification from one sample to another. Even if the fragment successfully amplifies, poor quality DNA can result in decreased signal or increased background fluorescent noise from the sequencing reactions.

For optimal results, use 10 to 20 ng/µL of template DNA with spectrophotometer absorbance ratios ($A_{260/280}$) between 1.8 to 2.0.

Factors affecting template quality

- **Type and amount of source material** – Influences the effectiveness and sensitivity of PCR amplification and the quality of sequencing results. The number of sequencing targets relative to the number of primer molecules can influence the efficiency and read-length of the sequencing reaction.
- **Contamination** – Can inhibit PCR amplification and cycle sequencing. Potential contaminants include:
  - Protein, RNA, or chromosomal DNA
  - Excess PCR primers, dNTPs, enzyme, and buffer components
  - Remaining salts, organic chemicals such as phenol, chloroform, and ethanol, or detergents.
  - Heparin—can partially or completely inhibit PCR amplification and cycle sequencing. The Dynabeads™ DNA DIRECT™ Blood Kit and the QIAamp™ Blood Kit (QIAGEN™, GmbH) successfully remove heparin from heparin blood samples, leaving genomic DNA ready for PCR amplification.

Note: Use a DNA isolation kit that is specifically designed for formalin-fixed, paraffin-embedded (FFPE) tissue and ensure that amplicon sizes are appropriate for the length of DNA fragment size that can be isolated.
Smaller amplicons compatible with FFPE-fragmented DNA can be designed using the free Primer Designer™ Tool found at **http://www.thermofisher.com/primerdesigner**.

Determining template quality and quantity

Use a spectrophotometer to determine DNA quality and to check for protein contamination. Optimum absorbance ratios ($A_{260/280}$) are between 1.8 and 2.0.

If DNA and/or RNA contamination is suspected, run your sample on an agarose gel. A single band should be present for high-quality DNA.

For DNA quantification, A_{260} values can be converted into µg/µL using Beer´s Law:

- Concentration of single-stranded DNA = A_{260} × 33 µg/µL.
- Concentration of double-stranded DNA = A_{260} × 50 µg/µL.

Optical density (OD) measurements are used to determine template concentration. Highly concentrated (OD >1.0) or very dilute (OD <0.05) DNA samples can lead to inaccurate OD measurements. Dilute or concentrate the DNA if needed to obtain an OD value between 0.05 to 1.

Note: OD measurement is not a reliable method to determine template concentration following enzymatic PCR purification protocols. Instead, estimate PCR product purity and concentration using an agarose gel or a flourescence-based method like the PicoGreen™ reagent for use on the Qubit™ quantification platform.

Primer guidelines

The method of primer purification and choice of M13 tailed- or non-tailed sequencing primers can have a significant effect on the ease of reaction set up and the quality of the sequencing data that is obtained in dye terminator cycle sequencing reactions.

- Use HPLC-purification for all primers to minimize cycle sequencing noise and provide longer sequencing reads.
- Use M13 sequencing primers to simplify the sequencing workflow when sequencing multiple PCR products and to reduce the loss of valuable 5′ unresolvable bases. With M13 sequencing primers, you make single forward and reverse reaction mixes, instead of multiple, primer-specific reaction mixes.

  Note: The M13 forward or reverse sequence must be incorporated at the 5′ end of the PCR primer to use the M13 sequencing primers.

Primer Designer™ Tool

Primer Designer™ Tool is a free online tool to search for the appropriate PCR/Sanger primer pair from a database of >650,000 pre-designed primer pairs for resequencing the human exome. Go to: **http://www.thermofisher.com/primerdesigner** for more information, including a direct link to purchase the designed primers online.

Prepare and store primers

1. Resuspend all PCR and sequencing primer stocks at 100 µM concentration in DNA buffer (10 mM Tris/0.1 mM EDTA, pH 8.0) and store them at –20°C.
2. Create individual amplicon-specific PCR primer pools of 0.8 µM PCR primers using UltraPure™ DNase/RNase-Free Distilled Water to minimize excess salt contribution that can inhibit subsequent reactions. Store working solutions at –20°C.

Amplify the DNA template with AmpliTaq Gold™ 360 Master Mix

Set up the PCR reaction

1. Completely thaw the AmpliTaq Gold™ 360 Master Mix and store on ice.

   Note: Store reagents at 4°C after first use.
2. Vortex the tubes for 2 to 3 seconds, then centrifuge briefly (2 to 3 seconds) with a benchtop microcentrifuge to collect contents at the bottom of the tubes.

3. Prepare the reaction mix:

   IMPORTANT! Change pipette tips after each transfer to avoid contamination of reagents, specimen, or amplicons.

Component	Quantity (1 well)	Quantity (96 well plate)[1]
AmpliTaq Gold™ 360 Master Mix	5 µL	528 µL
UltraPure™ DNase/RNase-Free Distilled Water	1 µL	106 µL
Total volume	**6 µL**	**634 µL**

[1] Includes 10% additional volume.

   Note: Store on ice until ready for use.

4. Vortex the tubes for 2 to 3 seconds, then centrifuge briefly (2 to 3 seconds) with a benchtop microcentrifuge.

5. Label a plate "PCR plate" and add the following, in order:

Component	Quantity
Reaction mix	6 µL
DNA template (10ng)	1 µL
Pooled PCR primers (0.8 µM each)	3 µL

   IMPORTANT! Change pipette tips after each transfer.

6. Seal the plate with MicroAmp™ Clear Adhesive Film.

7. Vortex the plate for 2 to 3 seconds, then centrifuge in a swinging bucket centrifuge to collect contents to the bottom of the wells (5 to 10 seconds) at 1,000 × *g*.

   Note: Bubbles may be present within the wells, but do not adversely affect the reaction.

Run the PCR

1. Place the plate in a thermal cycler and set the volume.
2. Run the PCR with the following settings:

Parameter	Stage/step					
	Incubate	Cycling (35 cycles)			Final extension	Hold
		Denature	Anneal [1]	Extend [2]		
Temperature	95°C	95°C	58°C	72°C	72°C	4°C
Time	10 minutes	30 seconds	30 seconds	45 seconds	7 minutes	Hold until ready to purify.

[1] If your primer annealing temperatures are not between 60°C and 65°C, annealing conditions may need optimization. See "Primer Designer™ Tool" on page 5 for more information.

[2] Extension times may need to be lengthened for sequences over 700 bp. For more information, refer to the *AmpliTaq Gold™ 360 DNA Polymerase Protocol*.

3. Place the plate on ice or store the plate at 4°C until ready for treatment with ExoSAP-IT™ *Express* PCR Product Cleanup.

   Note: Place plates at –25°C to –15°C for longer-term storage.

Treat the amplicons with ExoSAP-IT™ *Express* PCR Product Cleanup

1. Remove the 96-well plate from the thermal cycler, then centrifuge in a swinging bucket centrifuge for 10 seconds at 1,000 x g.
2. Place the plate and the tube of ExoSAP-IT™ *Express* PCR Product Cleanup on ice.
3. Remove the MicroAmp™ Clear Adhesive Film.
4. Transfer 5 µL of each PCR product to a new 96-well plate.
5. Add 2 µL of ExoSAP-IT™ *Express* PCR Product Cleanup to each well.

   IMPORTANT! Change pipette tips between wells.

6. Label the plate "+ExoSAP-IT."
7. Seal the +ExoSAP-IT plate with MicroAmp™ Clear Adhesive Film.
8. Vortex the plate for 2 to 3 seconds, then centrifuge (5 to 10 seconds) at 1,000 × *g*.

9. Incubate the +ExoSAP-IT plate:

Parameter	Stage/step	
	Digest	ExoSAP-IT™ Inactivation
Temperature	37°C	80°C
Time	4 minutes	1 minute

10. Store the treated plate on ice for immediate use or at –20°C for longer term storage.

Run sequencing reactions using the BigDye™ Terminator v3.1 Cycle Sequencing Kit

Set up the sequencing reactions

IMPORTANT! Protect dye terminators from light. Cover the reaction mix and sequencing plates with aluminum foil before use.

1. Completely thaw the contents of the BigDye™ Terminator v3.1 Cycle Sequencing Kit and your primers, then store on ice.

2. Vortex the tubes for 2 to 3 seconds, then centrifuge briefly (2 to 3 seconds) with a benchtop microcentrifuge to collect contents at the bottom of the tubes.

3. Label microcentrifuge tubes "forward" and "reverse", then add the following components to each tube:

   IMPORTANT! Change pipette tips after each transfer.

Component	Quantity			
	Forward reaction mix		Reverse reaction mix	
	1 reaction	96-well plate [1]	1 reaction	96-well plate[1]
BigDye™ Terminator v3.1 Ready Reaction Mix	2 µL	211 µL	2 µL	211 µL
5x Sequencing Buffer	1 µL	106 µL	1 µL	106 µL
Deionized water (RNase/DNase-free)	3 µL	317 µL	3 µL	317 µL
M13 forward primer (3.2 µM)	1 µL	106 µL	—	—

Component	Quantity			
	Forward reaction mix		Reverse reaction mix	
	1 reaction	96-well plate [1]	1 reaction	96-well plate[1]
M13 reverse primer (3.2 µM)	—	—	1 µL	106 µL
Total volume	**7 µL**	**740 µL**	**7 µL**	**740 µL**

[1] Includes 10% additional volume.

Note: Store on ice and protect from light.

4. Vortex the tubes for 2 to 3 seconds, then centrifuge briefly (2 to 3 seconds) with a benchtop microcentrifuge.

5. Label a new 96-well reaction plate "sequencing".

6. Place the "PCR + ExoSAP-IT" plate on ice, then remove the MicroAmp™ Clear Adhesive Film.

7. For each reaction, add the following, in order:

Component	Quantity
Reaction mix	7 µL
Purified PCR product (ExoSAP-IT™ *Express* PCR Product Cleanup)	3 µL

IMPORTANT! Change pipette tips after each transfer.

Note: Use an 8-tip multi-channel P10 pipette, if available, for amplicon transfer.

8. Seal the plate with MicroAmp™ Clear Adhesive Film.

9. Vortex the plate for 2 to 3 seconds, then centrifuge in a swinging bucket centrifuge to collect contents to the bottom of the wells (5 to 10 seconds) at 1,000 × *g*.

Note: Bubbles may be present within the wells, but do not adversely affect the reaction.

Run the sequencing reactions

1. Place the prepared sequencing plate into the thermal cycler, set the reaction volume, then run with the following conditions:

Parameter	Stage/step				
	Incubate	Cycling (25 cycles)			Hold
		Denature	Anneal [1]	Extend	
Temperature	96°C	96°C	50°C	60°C	4°C
Time	1 minute	10 seconds	5 seconds	4 minutes [2]	Hold until ready to purify.

[1] If your primer annealing temperatures are not between 60°C and 65°C, annealing conditions may need optimization. See "Primer Designer™ Tool" on page 5 for more information.

[2] Shorter amplicons (<500bp) can be run with shorter extension times (for example 2 minutes).

Note: Cycle sequencing will complete in 2 to 2.5 hours.

2. Place the plate on ice or store at 4°C until ready to purify the reactions.

Purify the sequencing reactions

Salts, unincorporated dye terminators, and dNTPs in sequencing reactions obscure data in the early part of the sequence and can interfere with basecalling.

The following methods are recommended for clean-up of cycle sequencing reactions:

- "Purify sequencing reactions with BigDye XTerminator™" on page 10
- "Purify the sequencing reactions with Centri-Sep™ plates" on page 11
- "Purify the sequencing reactions with ethanol/EDTA precipitation" on page 13

Purify sequencing reactions with BigDye XTerminator™

The following protocol takes approximately 40 minutes.

Note: Use disposable reagent reservoirs and an 8-channel P200 pipette, if available, to facilitate the clean-up process.

Note: If you use a 3730 DNA Analyzer, either MicroAmp™ Clear Adhesive Film or standard heat sealing techniques can be used.
This protocol describes plate sealing with MicroAmp™ Clear Adhesive Film.

1. Remove the BigDye XTerminator™ bead solution from 4°C storage and place on ice.

2. Vortex the bottle of BigDye XTerminator™ beads for 8 to 10 seconds before mixing with the SAM solution.

IMPORTANT! For effective BigDye XTerminator™ clean up, it is essential to keep the materials well mixed. Keep reagents on ice between pipetting steps.

3. Prepare the SAM/BigDye XTerminator™ bead working solution:

Component	Volume per 10 µL reaction	Volume per 96-well plate
SAM solution	45 µL	4.75 mL
BigDye XTerminator™ bead solution	10 µL	1.06 mL
Total volume	**55 µL**	**5.81 mL**

4. Remove the MicroAmp™ Clear Adhesive Film from the sequencing plate.

5. Dispense 55 µL/well of the SAM/BigDye XTerminator™ bead working solution to each sample.

   IMPORTANT! To mix thoroughly, pipette the solution up and down 3-4 times before each transfer. Re-mix solution after each dispense step.

6. Seal the plate using MicroAmp™ Clear Adhesive Film.

7. Vortex the 96-well plate for 20 minutes at 1,800 rpm (for the Digital Vortex-Genie™ 2).

8. In a swinging bucket centrifuge, centrifuge the plate at 1,000 × *g* for 2 minutes.

   Note: To store for up to 10 days, seal the plate with MicroAmp™ Clear Adhesive Film, and store at 4°C for capillary electrophoresis (CE) preparation or at –20°C until use. BDX plates can be stored at room temperature for up to 48 hours inclusive of time on the CE instrument.

Purify the sequencing reactions with Centri-Sep™ plates

The following protocol takes approximately 45 minutes (~25 minutes for purification and ~20 minutes for drying).

IMPORTANT! Do NOT skip the drying step in this procedure. Running samples that have not been dried will affect sequencing results.

Note: Individual Centri-Sep™ Spin columns can be used if few sequencing reactions need to be purified. Centri-Sep™ Spin columns must be hydrated for approximately 2 hours before use. Refer to the *DNA Sequencing by Capillary Electrophoresis Chemistry Guide* (Pub. no. 4305080) for more information.

1. Prepare 2.2% SDS (sodium dodecyl sulfate) in standard deionized water.

   Note: Store 2.2% SDS at room temperature. The SDS will precipitate at 4°C or below.

2. Briefly centrifuge the sequencing plate in a swinging bucket centrifuge (5 to 10 seconds) at 1,000 x g.

3. Remove the MicroAmp™ Clear Adhesive Film.

4. Prepare the SDS heat treatment:

Component	Volume
Sequencing reaction	10 µL
UltraPure™ DNase/RNase-Free Distilled Water	10 µL
2.2% SDS	2 µL
Total volume	**22 µL**

5. Vortex the plate for 2 to 3 seconds, then centrifuge (5 to 10 seconds) at 1,000 × *g*.

6. Perform the SDS heat treatment.

Parameter	Stage/step		
	Denature	Incubate	Hold
Temperature	98°C	25°C	4°C
Time	5 min	10 min	Hold

7. Prepare the Centri-Sep™ 96-well plate:

   Note: The Centri-Sep™ 96-well plates come pre-hydrated. The initial centrifugation step removes the hydration solution.

   a. Allow the plate to equilibrate to room temperature.

   b. Place the Centri-Sep™ 96-well plate in an empty 96-well plate.

   c. Centrifuge for 2 minutes at 1,500 x g to remove the hydration solution from the plate.

   d. Discard the plate with flow-through hydration solution.

   e. Place a new MicroAmp™ Optical 96-Well Reaction Plate beneath the prepared Centri-Sep™ 96-well plate to collect purified BigDye™ sequencing reaction product.

8. Briefly centrifuge the SDS heat-treated extension product plate in a swinging bucket centrifuge (5 to 10 seconds) at 1,000 x g and remove the MicroAmp™ Clear Adhesive Film.

9. Dispense 20 µL SDS heat-treated extension product to the corresponding Centri-Sep™ well. Dispense slowly into the center of the well (e.g. electronic pipette setting 4). Do not touch the sides of the well or the gel material.

10. Place a new 96-well collection plate beneath the Centri-Sep™ plate. Using a swinging bucket centrifuge, centrifuge the Centri-Sep™ plate containing the SDS heat treated sample for 2 minutes at 1,500 x g to collect purified sample.

11. Dry the sample in a vacuum centrifuge without heat or in low heat for 10 to 15 minutes or until dry.

12. Go to "Resuspend purified sequencing reactions" on page 14.

    Note: To store, seal the plate with MicroAmp™ Clear Adhesive Film, and store at 4°C for CE preparation or –20°C until use.

Purify the sequencing reactions with ethanol/EDTA precipitation

The following protocol takes approximately 90 minutes.

Note: This method produces a clean signal, but it can cause subtle loss of small molecular weight fragments.

IMPORTANT! Absolute ethanol absorbs water from the atmosphere, which gradually decreases its concentration and can affect sequencing results. Store appropriately and replace frequently.

1. Prepare a 125 mM EDTA solution from 0.5 M EDTA, pH 8.0.
2. Prepare 70% ethanol using absolute ethanol.

   Note: Replace every 2 weeks.

   IMPORTANT! Do NOT pre-mix 125 mM EDTA solution and absolute ethanol. This can cause precipitation of the EDTA.

3. Briefly centrifuge the sequencing plate in a swinging bucket centrifuge (5 to 10 seconds) at 1,000 x g.
4. Remove the MicroAmp™ Clear Adhesive Film from the plate.
5. Add the following in order:

Component	Volume
sequencing reaction (starting volume)	10 µL
125 mM EDTA solution	2.5 µL
absolute ethanol	30 µL
Total volume	**42.5 µL/well**

   IMPORTANT! Dispense the EDTA solution directly into the sample in each well before adding ethanol. If droplets are visible on the wall of the well, briefly centrifuge the plate to ensure that the EDTA mixes with the sequencing reactions.

6. Seal the plate with MicroAmp™ Clear Adhesive Film.
7. Vortex the plate for 2 to 3 seconds, then centrifuge (5 to 10 seconds) at 1,000 × *g*.
8. Incubate the plate at room temperature for 15 minutes.

   IMPORTANT! Timing of this step is critical.

9. Centrifuge the plate in a swinging bucket centrifuge at 1,870 x g (4°C) for 45 minutes.

   IMPORTANT! Proceed to the next step immediately. If there is a delay between steps, centrifuge the plate for an extra 2 minutes and proceed to the next step immediately.

10. Slowly remove the MicroAmp™ Clear Adhesive Film to prevent disruption of the pellet. Place 4 layers of absorbent paper into the centrifuge and carefully invert the plate onto the paper without dislodging the pellet. Centrifuge at 185 × g for 1 minute.

    Do not tip out liquid first. Do not tap plate to help with liquid removal.

11. Add 30 µL of 70% ethanol to each well.

12. Seal the plate with MicroAmp™ Clear Adhesive Film, then centrifuge at 1,870 × *g* (4°C) for 15 minutes.

    IMPORTANT! Proceed to the next step immediately. If there is a delay between steps, centrifuge the plate for an extra 2 minutes and proceed to next step immediately.

13. Slowly remove the MicroAmp™ Clear Adhesive Film to prevent disruption of the pellet. Place 4 layers of absorbent paper into the centrifuge and carefully invert the plate onto the paper towel without dislodging the pellet. Centrifuge at 185 × g for 1 minute.

    Note: Do not tip out liquid first. Do not tap plate to help with liquid removal.

14. Allow the plate to air dry, face up and protected from light, for 5 to 10 minutes at room temperature.

15. Go to "Resuspend purified sequencing reactions" on page 14.

    Note: To store, seal the plate with MicroAmp™ Clear Adhesive Film, and store, protected from light, at 4°C for CE preparation or –20°C until use.

Resuspend purified sequencing reactions

Resuspend samples purified with the Ethanol/EDTA and Centri-Sep™ methods.

Note: It is not necessary to resuspend samples purified with the BigDye XTerminator™ Purification Kit.

1. Remove the MicroAmp™ Clear Adhesive Film.

2. Resuspend dried samples in 10 µL of Hi-Di™ Formamide, then cover with MicroAmp™ Clear Adhesive Film.

   Note: Do not heat samples to resuspend.

3. Vortex thoroughly (5 to 10 seconds), then centrifuge in a swinging bucket centrifuge (5 to 10 seconds) at 1,000 x g.

   Note: Run samples as soon as possible after resuspension.

Run capillary electrophoresis

1. Remove the MicroAmp™ Clear Adhesive Film and replace with a 96-well plate septa.

   IMPORTANT! Plates sealed with heat seal film can be placed directly into the 3730/ 3730xl instruments. All other instruments require 96-well plate septa.

2. Load plates into the genetic analyzer.

3. Select the capillary length, number of capillaries and polymer type.

   Note: There is no default run module for POP-6™ when using the BigDye™ Terminator v3.1 Cycle Sequencing Kit on a 3500/3500xL Genetic Analyzer. Refer to the instrument user guide for creating run modules.

4. Select or create an appropriate run module according to your specific instrument user guide.

   IMPORTANT! Select a run module with a BDx prefix if you purified your sequencing reactions with BigDye XTerminator™. If your instrument does not contain BDx run modules, download them. Refer to the *BigDye XTerminator™ Purification Kit User Bulletin* (Pub. no. 4483510).

5. Select the injection time. Refer to your specific instrument user guide for information on using default settings or changing injection times.

6. Start the run.

Related documentation

Document	Publication number	Description
BigDye™ Terminator v3.1 Cycle Sequencing Kit User Guide	4337035	Describes the BigDye™ Terminator v3.1 Cycle Sequencing Kit hardware and software and provides information on preparing, maintaining, and troubleshooting the system.
Troubleshooting Sanger sequencing data	MAN0014435	This document provides guidance for the review of your data and troubleshooting tips for improving sequencing data quality.
DNA Sequencing by Capillary Electrophoresis Chemistry Guide	4305080	This chemistry guide is designed to familiarize you with Applied Biosystems™ genetic analyzers for automated DNA sequencing by capillary electrophoresis, to provide useful tips for ensuring that you obtain high-quality data, and to help troubleshoot common problems.

Document	Publication number	Description
BigDye XTerminator™ Purification Kit User Bulletin	4483510	This user bulletin provides: • A list of BigDye XTerminator™ Purification Kit run modules • Instructions for downloading and running the BDX Updater Utility to install the run modules • Instructions for running the BDX Updater Utility after you recalibrate the autosampler
BigDye XTerminator™ Purification Kit Quick Reference Card	4383427	This quick reference card provides instructions for BigDye XTerminator™ purification. In particular, it includes information on compatible plate vortexers and heat seal information for 3730 users.
Using an SDS/Heat Treatment with Spin Columns or 96-Well Spin Plates to Remove Unincorporated Dye Terminators	4330951	This protocol provides instructions for adding an SDS/heat treatment to the spin column and spin plate purification methods. This SDS/heat treatment effectively eliminates unincorporated dye terminators from your cycle sequencing reactions.

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    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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Revision history: Pub. No. MAN0017063

Revision	Date	Description
A.0	13 June 2017	New document.

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