PureQuant™ Th17 Assay

Catalog Number A43676

Pub. No. MAN0018288 Rev. C.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**.

Product description

The Applied Biosystems™ PureQuant™ Th17 Assay is a TaqMan™ qPCR-based assay that measures genomic DNA demethylation at a unique site of IL-17A gene, that in turn serves as an identifier of Th17 cells in a heterogeneous population. Genomic DNA isolated from cells is first subjected to bisulfite conversion followed by PCR amplification and finally qPCR using methylation specific primers. The assay utilizes Standards for estimating copy number. Reference serves as control. Each 96-well qPCR plate can accommodate from 1 to 7 test samples.

Contents and storage

Table 1 PureQuant™ Th17 Assay, (Cat. No. A43676)[1]

Contents	Amount	Storage
Dynabeads™ SILANE Genomic DNA Kit • Lysis Binding Buffer • Dynabeads™ magnetic beads • Wash Buffer 1 • Wash Buffer 2 • Elution Buffer	**1 Box**	4°C
PureQuant™ qPCR Master Mix	1 × 2 mL	4°C
PureQuant™ qPCR Bisulfite Conversion Reagents • PureQuant™ Ammonium Bisulfite • PureQuant™ THFA	**1 Box** 4 × 2.5 mL 1 × 3.5 mL	RT
PureQuant™ Th17 Module • PureQuant™ High Copy Standard A • PureQuant™ Reference Genomic DNA • Lambda DNA • TaqMan™ Th17 TpG Assay • TaqMan™ Th17 CpG Assay • PureQuant™ Th17 PCR Primer	**1 Box** 1 mL 6 µg 1.7 mL 200 µL 200 µL 40 µL	–20°C
PureQuant™ Hot Start PCR Master Mix • PureQuant™ Hot Start PCR Master Mix • Water, Nuclease-free	**1 Box** 0.5 mL 1.25 mL	–20°C

[1] PureQuant™ Th17 Assay is only available as a complete kit. Individual components are not sold separately.

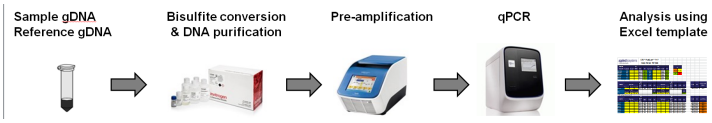
Required material not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Reagents	
PureLink™ Genomic DNA Mini Kit	K1820-01
2-Propanol	MLS
Ethanol	MLS
TE, pH 8.0	MLS
Instruments and equipment	
HulaMixer™ Sample Mixer	15920D
DynaMag™-2 Magnet	12321D
Eppendorf™ ThermoMixer™ C and SmartBlock™ 2 mL	Eppendorf™; 5382000023 and 5362000035
Eppendorf™ Safe-Lock Tubes, 2.0 mL	Eppendorf™; 022363344
Real Time-PCR Machine	MLS

IMPORTANT! The performance of this assay has been validated using a Eppendorf™ ThermoMixer™ C temperature control device. A dry heat block can be used as an alternative. However, use of the Eppendorf™ ThermoMixer™ C is recommended for most consistent results.

Assay overview



Guidelines to isolate genomic DNA and prepare samples

- Use the 2 mL Safe-Lock tubes for sample preparation.
- Use the Elution Buffer supplied with the Dynabeads™ SILANE Genomic DNA Kit.
- Do not add water into the Eppendorf™ ThermoMixer™ C with SmartBlock™ wells or heat block.

Isolate genomic DNA and prepare sample

1. Isolate genomic DNA from 1–2 × 10^6 cells using PureLink™ Genomic DNA Mini Kit and check the purity of gDNA using a spectrophotometer or NanoDrop™ spectrophotometer.

   The $OD_{260/280}$ and $OD_{260/230}$ of your gDNA should fall within the specified ranges:

   ($OD_{260/280}$:1.7–2.0 and $OD_{260/230}$:1.5–2.4)

2. Prepare Sample and Reference in 2 mL tubes according to the Table:

Item	Amount/volume of DNA	Make up the volume w/ Elution Buffer to
Sample	400–1200 ng	142 µL
PureQuant™ Reference Genomic DNA[1]	1000–1200 ng	142 µL

[1] Take 1 µL PureQuant™ Reference Genomic DNA and estimate the concentration using TE (pH 8.0) as blank. Expected concentration range between 150–250 ng/µL).

3. Incubate at 56°C for 5 min with gentle shaking (900 rpm) with a Eppendorf™ ThermoMixer™ C with 2 mL SmartBlock™ or (dry bath with 2 mL block).

4. Briefly spin down the samples.

5. Adjust the Eppendorf™ ThermoMixer™ C temperature to 80°C for the bisulfite conversion.

Guidelines for bisulfite conversion

- During bisulfite conversion, follow the recommended incubation times as over-incubation or under-incubation will impact the assay result.
- Bring Lysis Binding Buffer and Dynabeads™ magnetic beads to room temperature before use.
- Make a homogeneous suspension of Dynabeads™ magnetic beads before use.

Perform bisulfite conversion

1. Add 270 µL ammonium bisulfite and 90 µL THFA to the Samples, Calibrator, and Reference.

   Vortex thoroughly and briefly spin down the samples.

2. **Eppendorf™ ThermoMixer™ C**: Incubate at 80°C for 45 minutes with gentle shaking (900 rpm). Briefly centrifuge to spin down the samples and allow them to cool down to room temperature before proceeding to the next step.

   OR

   Heat block: Incubate at 80°C for 45 minutes with intermittent vortexing for 1–2 seconds at 4.5-minute intervals. Minimize any delay that may occur during vortexing. Briefly spin down the samples at the end of the 45 minute incubation. Allow samples to cool down to room temperature before proceeding to the next step.

3. Add 870 µL Lysis Binding Buffer and 105 µL Dynabeads™ magnetic beads from the (Dynabeads™ SILANE Genomic DNA Kit) to each reaction.

4. Mix thoroughly by vortexing and briefly spin down the tubes.

5. Add 570 µL of 2-propanol and vortex thoroughly.

6. Incubate at room temperature for 7 minutes on a rotating mixer or HulaMixer™ Sample Mixer under constant vertical rotation (50 rpm).

7. Briefly spin down the tubes and place them in the DynaMag™-2 Magnet for 5 minutes.

8. While in the magnetic rack carefully pipette off the supernatant without transferring beads.

   Note: The beads contain the DNA.

Guidelines to purify DNA

- Bring Wash Buffers and Elution Buffer from Dynabeads™ SILANE Genomic DNA Kit to room temperature before use.
- Vortex sufficiently to ensure that beads are resuspended completely in each wash.
- Remove any residual Wash Buffer 2 before drying the beads at 65°C.
- Add ethanol and isopropanol to the wash buffers as recommended on the bottles.

Purify DNA

1. Remove the tubes from the magnetic rack and add 900 µl of Wash Buffer 1.

2. Vortex at maximum setting to make sure that beads are resuspended completely.

   Briefly spin down the tubes.

3. Place the samples in the magnet for 3 minutes.

4. While the tubes are in the magnet, remove the supernatant without transferring beads.

5. Repeat the washing (steps 1–3) with Wash Buffer 1.

6. Add 900 µL of Wash Buffer 2.

7. Resuspend the beads completely by vortexing.

   Briefly spin down the tubes.

8. Place the samples in the magnet for 3 minutes.

9. While the tubes are in the magnet, remove the supernatant without transferring beads.

10. Add 400 µL Wash Buffer 2.

11. Resuspend the beads completely by vortexing.

    Briefly spin down the tubes.

12. Place the samples in the magnet for 3 minutes.

13. While the tubes are in the magnet, remove the supernatant without transferring beads.

    Briefly spin down the tubes.

14. Place the samples in the magnet for 3 minutes.

15. Remove any residual Wash Buffer and remove the tubes from the magnetic rack.

16. Dry the beads with the lid open at 65°C for 15 minutes in a Eppendorf™ ThermoMixer™ C (or a dry-bath).

17. Add 25 µL Elution Buffer.

18. Incubate at room temperature for 7 min under constant shaking (1400 rpm) in the Eppendorf™ ThermoMixer™ C, or use a vortex with a foam adapter at moderate speed.

19. Place the tubes in the magnet for 2 minutes.

20. Carefully transfer the eluate (15 µL) to a fresh tube. The eluate contains bisulfite-converted DNA that is used in subsequent qPCR.

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Preamplify DNA

1. Transfer 2 µL of bisulfite converted DNA into PCR tubes/strips.

2. Set up an equal volume of water in a single reaction as a preamplification negative control.

3. Setup preamplification reaction master mix for all samples and controls according to the Table.

Reagent	Amount
DNA	2 µL
Water, Nuclease-free	9.5 µL
PureQuant™ Hot Start PCR Master Mix	12.5 µL
PureQuant™ Th17 PCR Primer	1 µL
Total	**25 µL**

4. Add 23 µL preamplification reaction master mix to each reaction tube, vortex briefly and centrifuge.

5. Perform the preamplification protocol using a thermal cycler with a heated lid.

Table 2 Preamplification reaction thermal cycler conditions

Step	Time	Temp	Cycles
Pre-incubation	35 min	95°C	1X
Denaturation	1 min	95°C	12X
Annealing	45 sec	55°C	
Elongation	30 sec	72°C	
Final elongation	10 min	72°C	1X
Hold	Indefinite	4°C	

6. Briefly centrifuge the PCR tubes containing the preamplification reactions.

7. Transfer 2 µL of each reaction into a clean PCR tube and dilute with 78 µL water (1:40).

Guidelines for setting up qPCR

- Refer to instrument user manual for instructions programming qPCR run with Standard Curve.

IMPORTANT! Assign all the standard dilutions as "Standard." Assign samples , negative control and reference as "Unknown"; and no template control as "NTC or N" in triplicate.

IMPORTANT! While assigning wells for each standard, provide copy number that are specified in the Final Copy Number column (see “Make standard dilutions”).

- Use the same six standard dilutions for both Th17 TpG and Th17 CpG Standards.
- All Samples, Standards and Controls are run in triplicate.

Make standard dilutions

Prepare a full panel of six standard dilutions by serially diluting the PureQuant™ High Copy Standard A starting at 31250 copies/3 µL according to the Table. The same standard dilutions are used for both Th17 TpG and Th17 CpG in qPCR. Store the standard dilutions at –20°C.

Initial plasmid Copy Number/3 µL	Volume	Diluent DNA [1]	Final Copy Number per 3 µL	Label
31250	1000 µL	—	31250	STD#1
31250	200 µL	800	6250	STD#2
6250	200 µL	800	1250	STD#3
1250	200 µL	800	250	STD#4
250	200 µL	800	50	STD#5
1250	30 µL	1200	30	STD#6[2]

[1] 10 ng/µL Lambda DNA in TE (10 mM Tris, 1 mM EDTA, pH 8.0)
[2] Use STD#3 to prepare STD#6.

Prepare PCR plate

1. Prepare the qPCR Master Mix Cocktail in two separate tubes, one each for TpG and CpG, excluding Template DNA according to the Table:

Reagent	Amount
Template DNA	3 µL
Lambda DNA (50 ng/µL in TE, pH 8.0)	1 µL
TaqMan™ assay	0.5 µL
Water, Nuclease-free	0.5 µL
PureQuant™ qPCR Master Mix	5 µL
TOTAL	**10 µL**

2. Load the templates first and then add the 7 µL master mix cocktail.

	Th17 TpG						Th17 CpG					
	1	2	3	4	5	6	7	8	9	10	11	12
A	STD-1_TpG			Sample#1			STD-1_CpG			Sample#1		
B	STD-2_TpG			Sample#2			STD-2_CpG			Sample#2		
C	STD-3_TpG			Sample#3			STD-3_CpG			Sample#3		
D	STD-4_TpG			Sample#4			STD-4_CpG			Sample#4		
E	STD-5_TpG			Sample#5			STD-5_CpG			Sample#5		
F	STD-6_TpG			Sample#6			STD-6_CpG			Sample#6		
G	Reference			Sample#7			Reference			Sample#7		
H	Pre-amp Negative ctrl			NTC			Pre-amp Negative ctrl			NTC		

Figure 1 A representative 96-well plate layout illustrating Th17 assay set up

Note: All Samples, Standards and Controls are run in triplicate.

3. Seal the plate with film and briefly centrifuge before placing into the qPCR instrument.

4. Select FAM or equivalent as dye and Non Fluorescent Quencher (NFQ) or equivalent as quencher.

Table 3 qPCR cycle setup parameters

Step	Time	Temp	Cycles
Pre-incubation	10 min	95°C	1X
Amplification	15 sec	95°C	50X
	1 min	61°C	
Cooldown	5 sec	42°C	1X

5. Run qPCR using the cycle set up parameters listed in Table 3.

IMPORTANT! See "Guidelines for setting up qPCR" for instructions on setting up the qPCR standard curve and other controls.

6. After the qPCR run is complete, execute analysis on the qPCR instrument to calculate: Ct Average, Ct Standard Deviation, and Copy Number. Export data in .txt or .xlsx format.

7. Analyze data using PureQuant™ Th17 Assay Microsoft™ Excel™ Analysis Template. Download Analysis Template from **thermofisher.com/order/catalog/product/A43676**.

   The analysis template has three tabs: Tab#1 contains instructions and guidance on how to perform data analysis, Tab#2 is the Analysis Template, and Tab#3 contains an Example.

Limited product warranty

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