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Products

MyTREC™ Sensi Duplex TREC/ Beta Actin Real-Time qPCR Assay Kit (Human)

- **GP-D3012096 …… 96 x 20 µl Reactions**
- **GP-D3012045 …… 45 x 20 µl Reactions**

MyTREC™ TREC Singleplex Real-Time qPCR Assay Kit (Human)

- GP-T10120100 ……. 100 x 20 µl Reactions
- GP-T10120500 ……. 500 x 20 µl Reactions

MyTREC™ Beta Actin Singleplex Real-Time qPCR Assay Kit (Human)

- GP-A20120100 ……. 100 x 20 µl Reactions
- GP-A20120500 ……. 500 x 20 µl Reactions

Rhesus macaque (*Macacus mulatta*)

TREC Singleplex Real-Time qPCR Assay Kit

- GP-MM50120100 ……. 100 x 20 µl Reactions
- GP-MM50120500 ……. 500 x 20 µl Reactions

Beta Actin Singleplex Real-Time qPCR Assay Kit

- GP-MM60120100 ……. 100 x 20 µl Reactions
- GP-MM60120500 ……. 500 x 20 µl Reactions

MyTREC™ Sensi Duplex TREC/Beta Actin Real-Time qPCR Assay Kit

For Research Use Only

Products' Specifications

- GP-D3012096 96 x 20 µl Reactions
- GP-D3012045 45 x 20 µl Reactions

GP-D3012096 and GP-D3012045 are a TREC / Beta Actin Duplex Assay Kit and measures both TREC and Beta Actin counts in a single reaction.

The Kit is optimized for 20 µl reactions only.

GP-D3012096 has components to run a total of 96 x 20 ul reactions that includes reactions for Calibrators (Standards), Non Template Control (NTC), Positive Control and Test Samples. Also, the Calibrators are in sufficient quantity for one Calibration curve, set up in triplicate.

GP-D3012045 has components to run a total of 45 x 20 ul reactions that includes reactions for Calibrators (Standards), Non Template Control (NTC), Positive Control and Test Samples. Also, the Calibrators are in sufficient quantity for one Calibration curve, set up in triplicate.

Storage and Stability

All components of the kit should be stored at -20 to -30 deg C (constant temperature non-frost-free freezer). Avoid repeat freeze-thaws. Avoid exposure of components (probes, ROX®, Gene Expression Master Mix containing ROX®) to light. With proper storage and handling, the full activity of the kit is retained until the expiry date.

Description

The MyTREC™ Sensi Duplex TREC/Beta Actin Real -Time qPCR Assay Reagent Kit has been designed and developed for sensitive, accurate and simultaneous quantification of *Human Signal Joint* **(δRec-ΨJα Sj**) TRECs (T-Cell Receptor Excision Circles) and Beta Actin (endogenous reference) in a single reaction, by **Absolute Quantification Method generating Calibration / Standard Curves**. The proprietary Calibrators are "Ready-To-Use" and are serially diluted in a stabilizing diluent. The kit is formulated with TaqMan® dual-labeled fluorescent probes and a Gene Expression Master Mix containing antibody-mediated hot-start DNA Polymerase, Enhancers, Stabilizers, dNTPs, etc. A separate ROX® reference dye stock aliquot is provided for optional use and to enable instrument compatibility. **The kit is optimized for standard mode on fast qPCR instruments and standard cycling conditions on standard qPCR machines.**

Components

	Volumes µl GP-D3012096 / GP-D3012045
• Calibrators, **C** (contains both TREC & Beta Actin)	15 **/** 15
• Gene Expression Master Mix, 2x, **MM**	1000 / 500
• ROX® stock solution (light-sensitive), **ROX**	45 **/** 25
• Primers / Probe, TREC (light-sensitive), 40x, **Tp/p**	55 **/** 30
• Primers / Probe, Beta Actin (light-sensitive), 40x, **Ap/p**	55 / 30
• Reaction Enhancer, 20x, **RE**	110 / 55
• Positive Control (contains both TREC and Beta Actin) **PC**	15 /15
• Nuclease-Free Water, **NFW**	1000 / 1000

Note: The Primers / Probe (Tp/p and Ap/p) are at 40x concentration

Experiment Method

Real -Time qPCR Assay by Absolute Quantification using a Calibration / Standard curve

Calibrators

Calibrator copy numbers / 4 µl

Calibrator	TREC copies	Beta Actin Copies
C1	25	125
C2	10^2	$5x10^2$
C3	10^3	$5x10^3$
C4	10^4	$5x10^4$
C5	10^5	$5x10^5$
C6	10^6	$5x10^6$

- Note: Have designated areas for Test Sample preparation, PCR set up and PCR run
- Note: Use a dedicated clean PCR area / PCR Workstation to set up the PCR.
- Note: Use sterile nuclease-free low adhesion barrier tips / other supplies for the PCR.
- Note: Before you thaw the MyTREC™ Kit components, prepare your Test Samples and program the PCR Instrument for the run.

Protocol

1. Process your test samples for genomic DNA extraction and suspend the DNA in NFW. Freeze them until use.

2. Set up your Real-Time qPCR Instrument for the run, according to instrument's operator manual. The fields may vary with instrument.

- Set up a new experiment: Standard Curve (by Absolute Quantification), TaqMan® reagents, Standard Mode or Standard Cycling Conditions.
- **Set up the qPCR plate by defining the targets / samples to both TREC and Beta Actin Reporters / Quenchers:**

FAM™ (Reporter, TREC) / NFQ-MGB (Quencher) **AND**
VIC® (Reporter, Beta Actin) / NFQ-MGB (Quencher)
Passive Reference Dye = ROX® (optional, depending on your instrument)

Note: Check if your PCR instrument supports FAM™ / VIC® reporters and/or requires calibration.

Note: If your PCR instrument does not give the option for choosing the quencher NFQ-MGB, or a Non-Fluorescent Quencher, check the operator's manual or call the manufacturer to know the alternative quencher parameters that could work equally well.

- Set up plate by assigning targets and samples to the wells in the plate as Standards, NTC (Non-Template Control), Unknowns. Assign Calibrator copy numbers (both TREC and Beta Actin) for each of the Calibrator wells.

Note: For Calibrator copy numbers, see page 3

- Set up qPCR cycling program on your PCR instrument as in Table 1:

Table 1: Cycling Protocol

Step	Cycles	Temperature	Time (hr:min:sec)
Polymerase Activation	1	95 deg C	00:03:00
Amplification	40		
Denaturation		95 deg C	00:00:30
Annealing/Extension		60 deg C	00:01:00
Hold	1	4 deg C	24:00:00

3. Thaw the MyTREC™ Kit Reagents on ice (protect light sensitive components from light)

4. Give a gentle / brief vortex to thawed components and centrifuge briefly to collect the contents at the bottom of the tubes. During the reaction set up, if the components are sitting idle for a while, tap the tube / contents to mix and spin briefly.

5. Add ROX® reference dye to the Gene Expression Master Mix (Table 2). Check "Instrument Compatibility" on page 11 to know if your instrument is a high / low ROX® system. For instruments not listed, please check with your manufacturer.

Table 2: Amount of ROX® dye to add to the Gene Expression Master Mix

	ROX® Dye Volume (µl)	
	High ROX® dye system	Low ROX® dye system
To 1ml Gene Expression Master Mix (GP-D3012096)	40	4
To 0.5 ml Gene Expression Master Mix (GP-D3012045)	20	2

6. Make Reaction Mix (*Make Reaction Mix just before use. It cannot be stored*).

- Make enough Reaction Mix for the number of reactions needed plus 2-4 additional reactions to account for the pipetting errors.
- We recommend setting up reactions in triplicate for statistical significance. Set up reactions for Calibrators, NTC, Positive Control and Test Samples.
- Set up a 1 x 20 µl Reaction Mix as in Table 3. Multiply the volumes by the "number of reactions" to scale up the Reaction Mix preparation.

Table 3: Reaction Mix set up.

Gene Expression Master Mix (2x)	10 µl
Primers / Probe, TREC (40x)	0.5 µl
Primers / Probe, Beta Actin (40x)	0.5 ul
Reaction Enhancer (20x)	1 µl
Nuclease-Free Water	4 µl
	16 µl

7. Dispense 16 µl aliquots of Reaction Mix into the wells of the qPCR plate that is compatible with your Real-Time PCR Instrument.
8. Add DNA template (see below) and make up the final reaction volume in the well with NFW (as needed) to 20 µl. Pipette up and down twice gently to mix contents.

   - Calibrator wells : 4 µl Calibrator DNA (**contains TREC & Beta Actin copies**)
   - NTC wells : 4 µl NFW
   - Positive Control wells : 4 µl PC (**contains both TREC & Beta Actin copies**)
   - Test Samples : up to 4 µl (we recommend using 100 pg – 500 ng DNA)

9. Seal the plate with optically transparent film.

10. Spin the plate briefly to settle the contents down and remove any air bubbles / or bring air bubbles to the top.

11. Run the qPCR

    - Go to the experiment / cycling protocol (as in Table 1) you programmed previously on your PCR Instrument and start the run.

Note: Make sure the run is initiated before you walk away from the instrument.

12. **Save the experiment: Always save the experiment, prior to any analysis. And save the analyzed experiment as a separate file.**

13. Reading the results

    - The absorption range of fluorophores is seen in Table 4. View results in the channel specific for the FAM™ / VIC® dye.

Table 4: Absorption range of fluorophores

Dye	Absorption Max (nm)	Emission Max (nm)
6-FAM™	~ 495	~ 515
VIC®	~ 535	~ 555
ROX®	~ 573	~ 602

14. Analysis guidelines

    - Perform data analysis as described in the instrument's operator's manual
    - Evaluate the individual calibration curves and amplification plots for TREC and Beta Actin targets.
    - Review the Slope, Amplification Efficiency, Correlation Coefficient (R^2) and individual Threshold Cycle (C_T) values of Calibrators.
    - Slope / Amplification Efficiency Values: A slope close to -3.3 indicates optimal and 100% amplification efficiency.

- R^2 Value (Correlation Coefficient): An R^2 value > 0.99 is desirable. The R^2 value is a measure of the closeness of fit between the regression line and the individual C_T data points of the Calibrators.
- Evaluate Amplification Plots to examine / identify any irregular amplification curves.
- Evaluate threshold and baseline values for the run. Make adjustments to threshold and baseline values if necessary.
- Some instrument software / experiments may need manual baseline and threshold settings for accurate analysis.
- Baseline: Baseline setting includes a range of lower and upper baseline settings to obtain optimal background subtraction. Based on TaqMan® probe chemistry, we recommend using **auto baseline** setting. Typically, the auto baseline feature finds the best algorithm to optimally subtract the noise on a well-to-well basis (see advanced settings).
- Automatic baseline sometimes can fail: In certain cases, if a sample produces noise at the beginning of the run (Fig.1A), the instrument's software fails to distinguish noise from true amplification. This results in an incorrect auto baseline setting and insufficient background signal subtraction causing the affected sample to assume a sigmoidal amplification curve (Fig.1A). At this point, disable the auto baseline setting. The software then shifts from auto to preset **manual default setting** values of 3/15 for start and end cycles (Fig.1B)

Figure 1A: Auto Baseline

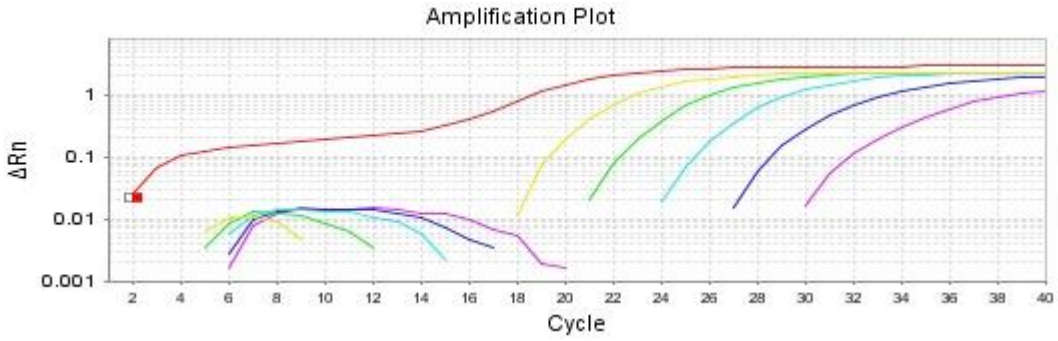
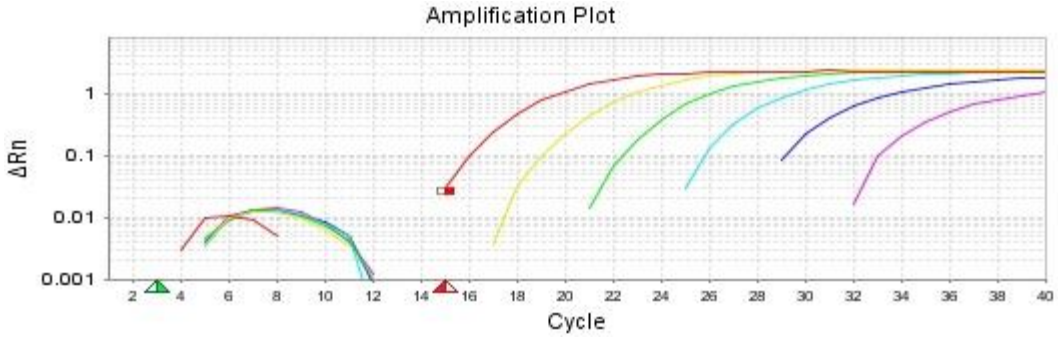


Figure 1B: Manual Baseline



- Threshold: Set threshold correctly. Too low or too high threshold increases the standard deviation between replicate C_T values.
- **Auto Threshold** is set by the software. Verify if your auto threshold is set correctly and meets the following criteria: (a) is set in the geometric / exponential phase of the amplification plot; (b) increases the precision of replicate Calibrators indicated by reduced standard deviation between replicate C_T values and (c) maximizes the sensitivity of the assay across all orders of magnitude.
- **Manual Threshold:** Disable auto threshold and move the threshold line to fulfill the parameters mentioned above.
- Reevaluate the Standard Curves and Amplification Plots and remove any outlier Calibrator points (1-3 out of a total 18 Calibrator measurements) by omitting them from analysis.

15. Typical Results of MyTREC™ Sensi Duplex TREC/Beta Actin Real-Time qPCR Kit on StepOne™ Real-Time PCR System

Figure 2A

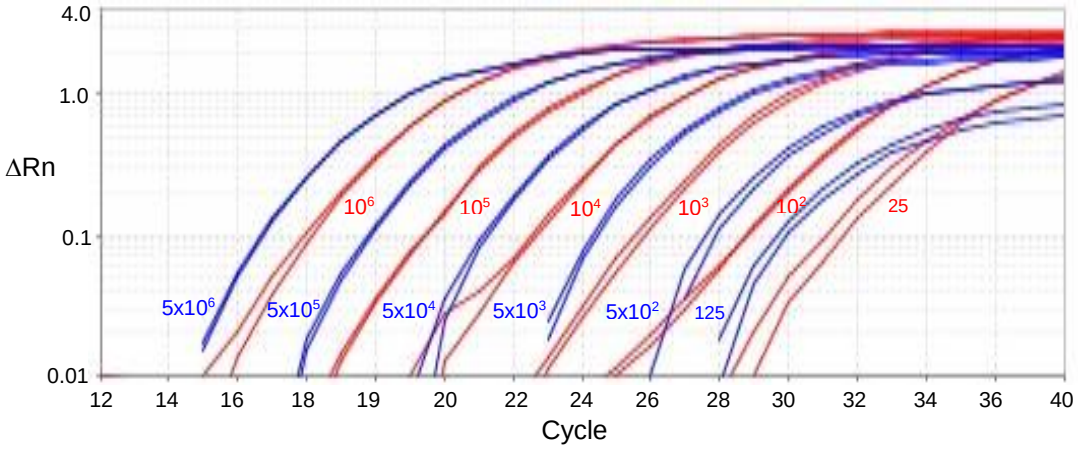
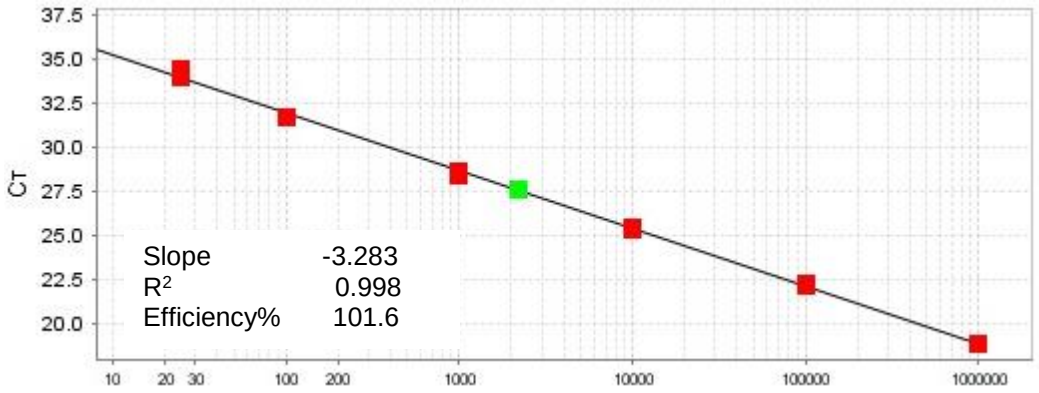


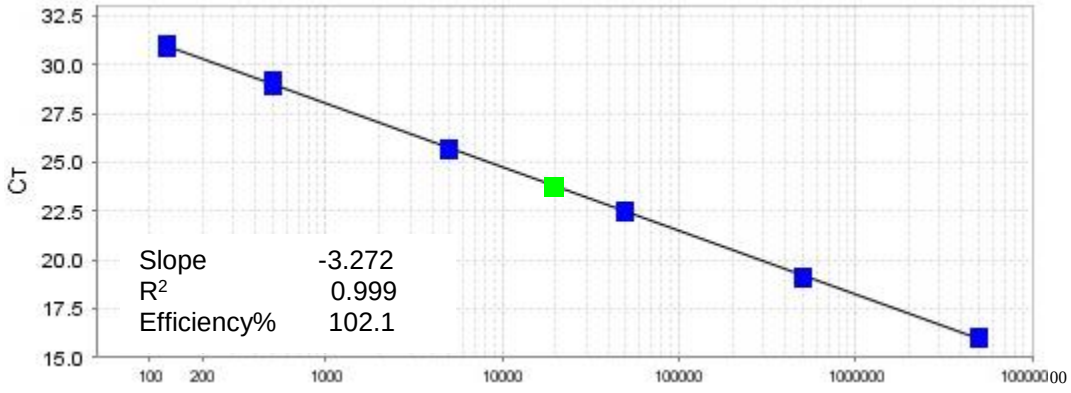
Figure 2A, **2B**, **2C**: MyTREC™ Sensi Duplex TREC/Beta Actin Real-Time qPCR Assay.
Figure 2A: The TREC and Beta Actin Calibrators (range of ~6-$\log_{10}$) were amplified in the same reaction tube with Gene Expression Master Mix, primers and probes (TaqMan® FAM™ probe for TREC and TaqMan® VIC® probe for Beta Actin). The reactions were performed in duplicate on StepOne™ Real-Time PCR Instrument and data was analyzed using StepOne™ Software v2.3. Amplification plots (Fig.2A) of TREC Calibrators (red) and Beta Actin (blue) are shown along with the copy numbers of each Calibrator.
Figure 2B: Standard Curve and linear regression statistics of the TREC Calibrators (red squares) are shown in Fig.2B. The green square represents the Positive Control.
Figure 2C: Standard Curve and linear regression statistics of the Beta Actin Calibrators (blue squares) are shown in Fig.2C. The green square represents the Positive Control.

Figure 2B



Log Copy Number (TREC)

Figure 2C



Log Copy Number (Beta Actin)

16. Evaluating the Test Samples' Real-Time qPCR run

- Evaluate the amplification plots of all Test Samples. Any atypical plot should be rejected and retested.
- The Test Sample C_T and its analyte (TREC and Beta Actin) copy number is automatically obtained by the software from the standard curve plots.
- Analyze the Test Sample data as seen in Table 5.

Table 5: Data Analysis

Test Sample	Positive Control	NTC	Result
No Amplification	C_T<35	Negative	TREC / Beta Actin Negative
Amplification Signal	C_T<35	Negative	TREC / Beta Actin Positive
No Amplification	Not detectable	Negative	PCR Failure
Amplification Signal	Amplification Signal	Positive	PCR Contamination

- **Positive Control: The TREC copy number for the Positive Control falls approx. in the middle of the Calibration curve (Fig.2B), between 10^4 and 10^3 copies.**
  The Beta Actin copy number for the Positive Control falls approx. in the middle of the Calibration curve (Fig.2C), between $5x10^4$ and $5x10^3$ copies

17. Normalization of Data, TREC Measurements and Reporting Units
    Reference gene Beta Actin is used to normalize the DNA loads and remove any technical variations from the final calculations. To normalize the data for the Test Samples, obtain the linear quantity of TREC and Beta Actin counts and use the following correction factors to achieve the final TREC measurements. The TREC counts are reported in standard reporting units of either:

    - TREC copies / 1 million cells
    - TREC copies / 1 µg DNA

    Table 6: TREC Normalization / Reporting Calculations

Mean TREC counts (T) = Average of TREC counts of replicates
Mean Beta Actin counts (B) = Average of Beta Actin counts of replicates
Normalized TREC counts (T/B) = Mean TREC counts / Mean Beta Actin counts
TREC counts per 1 million cells = (T/B) x 2 x 10^6
TREC counts per 1 µg genomic DNA = (T/B) x 3.03 x 10^5

    - **If you wish to compare TREC measurements across samples, process / prep the samples similarly for genomic DNA and normalize the DNA loads used for the Real-Time PCR**.
    - Note: TREC measurements can also be reported per 1 ml blood. For a DBS spotting volume of 10-20 µl, a 3 mm punch ≈ 3 µl blood volume.
    - Note: A conversion factor of 6.6 pg of genomic DNA per cell and a ploidy of 2 (Beta Actin) was used in the above calculations.

18. Instrument Compatibility

Real-Time PCR Instruments

MyTREC™ Kits are compatible with various common Real-Time qPCR Instruments capable of recording FAM® and VIC® fluorescence. The following list is provided as a guideline for your reference. Call the manufacturer to ask about your instrument and know if your machine is compatible with a specific reporter dye and if it needs calibration for any particular dye. The list is not all inclusive.

High ROX

- Applied Biosystems 5700
- Applied Biosystems 7000
- Applied Biosystems 7300
- Applied Biosystems 7700
- Applied Biosystems 7900
- Applied Biosystems 7900HT
- Applied Biosystems 7900 HT Fast
- Applied Biosystems StepOne™
- Applied Biosystems StepOnePlus™

Low ROX

- Applied Biosystems 7500
- Applied Biosystems 7500 Fast
- Stratagene Mx3000P®
- Stratagene Mx3005P™
- Stratagene Mx4000™
- Applied Biosystems ViiA 7
- Applied Biosystems QuantStudio™
- Agilent AriaMx
- Douglas Scientific IntelliQube®
- QIAGEN Rotor-Gene Q

No ROX

- BioRad CFX
- BioRad iQ™
- BioRad Opticon™
- Roche LightCycler®