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Created: 08/04/10

HUMAN TREC ASSAY

Isolation of PBMCs

-As per standard procedures
-Draw blood (10ml is adequate)
-Isolate PBMCs on Ficoll or sort T cells by FACS/MACS (sorting is better)

Purification of genomic DNA

-As per standard procedures
-Easy DNA kit (Invitrogen)
-Need 1ug DNA from PBMCs or 250ng DNA from purified T-cells (1vial)

Standard dilutions of TREC and CCR5 plasmids

-human signal joint TREC in pCR Blunt plasmid from Daniel Douek (NIH)
1 plasmid=3888bp and 1bp=660Da or g/mole so 1bp=$1.096x10^{-21}$g
1 plasmid=$4.26x10^{-18}$g or $4.26x10^{-12}$ug
-human CCR5 in pC1 plasmid from Mike Bell (Mayo Clinic)
1 plasmid=6530bp and 1bp=660Da or g/mole so 1bp=$1.096x10^{-21}$g
1 plasmid=$7.16x10^{-18}$g=$7.16x10^{-12}$
-Take an O.D. reading of stock plasmid solutions @260nM and calculate concentration. 1 O.D. unit @260nm=50ug/ml.
-Make serial dilutions of TREC and CCR5 plasmids. Make 1 ml of standard at 1×10^6 plasmids/5ul. Dilute this standard 1:10 from $lx10^5$ plasmids to $lx10^{-2}$ plasmids. This has to be extremely accurate!!! Change pipette tips, increase volumes, (do whatever it takes to make this perfect).
-Perform a test PCR run on TREC and CCR5 standard dilutions. Remember to include samples with no plasmid. If you observe amplification of standards containing less than 1 plasmid, (1×10^{-1} or 1×10^{-2}) the standard curve is off. If the standard curve is accurate, save standards for future experiments. When making new standards, always check them against old standards.

Real Time PCR

-Performed in spectrofluorometric thermal cycler (ABI PRISM 7700, PE Applied Biosystems).
-sjTREC reactions contain 25pmol primers (forward CACATCCCITTCAACCATGCT , reverse GCCAGCTGCAGGGTTTAGG), 125 nM TaqMan probe (FAM-ACACCTCTGGTTTTTGTAAAGGTGCCCACT T AMRA-6.25pM), 1X TaqMan Universal PCR Master Mix (PE Applied Biosystems), and 50-400ng of template DNA in a total volume of 50ul.
-CCR5 reactions contain 25pmol primers (forward GTGTCAAGTCCAATCTATGACATCAA, reverse GCCTGCGATTTGCTTCACA), 125 nM TaqMan probe (FAM - TATTATACATCGGAGCCCTGCCAAAAAATCA-TAMRA-6.25pM), 1X TaqMan Universal PCR Master Mix, and 40-160ng of template DNA in a total volume of 50ul.

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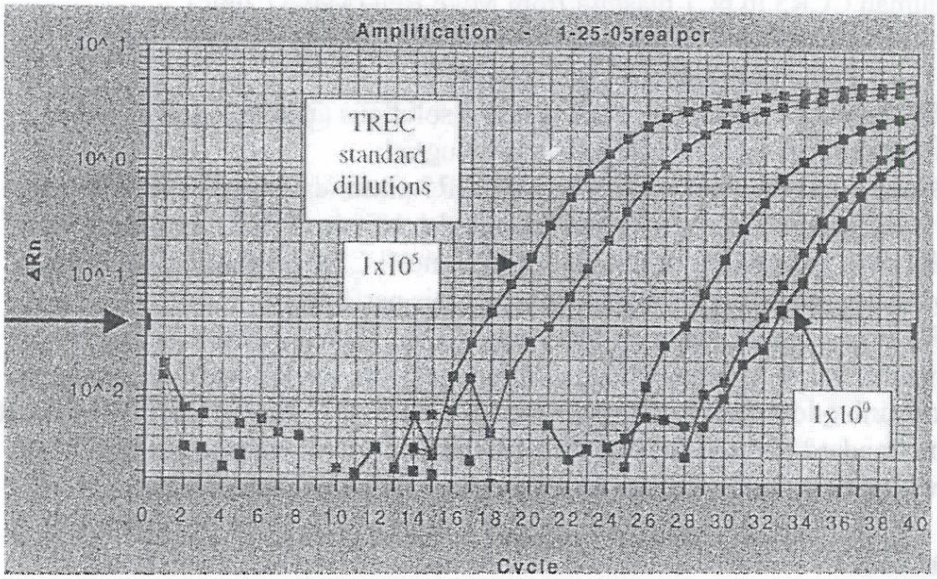
Thermal cycling conditions consist of a 2 minute incubation at 50°C, and an initial denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 10 seconds and 60°C for 1 minute.
-Run TREC and CCR5 standard curves in every experiment.
-Because O.D. readings on input DNA can be inaccurate, it is important to run each patient sample with a few different amounts of input DNA. The following concentrations work well: 0ng, 50ng, 100ng, 200ng, and 400ng.

Calculation of TRECs/PBL

-The thermal cycler plots cycle number versus fluorescence. The cycle threshold (Ct) represents the number of cycles it takes for a sample to amplify above background noise. It is sometimes necessary to adjust the threshold as shown below.



Threshold should be at arrow, so roughly equal spacing between curves at point of intersection with threshold

-For TREC and CCR5 standard dilutions, plot number of plasmids (copies) on a log scale versus cycle threshold (Ct).

CCR5 Copies	Ct
100,000	22.38
10,000	25.466
1,000	29.208
100	32.868
10	36.309

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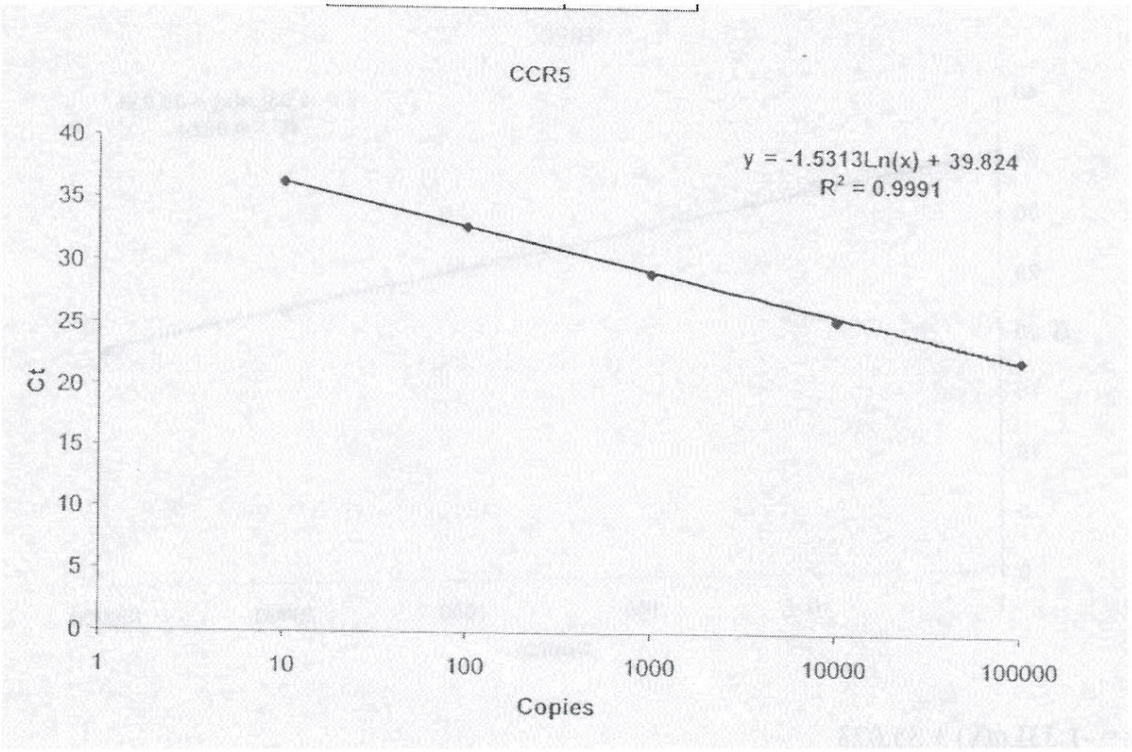

$y = -1.5313\ln(x) + 39.824$
$R^2 = 0.9991$

-Calculate the equation describing the curve. Excel will do this.

TREC Copies	Ct
100,000	19.329
10,000	22.686
1,000	26.128
100	29.343
10	32.45
1	34.264

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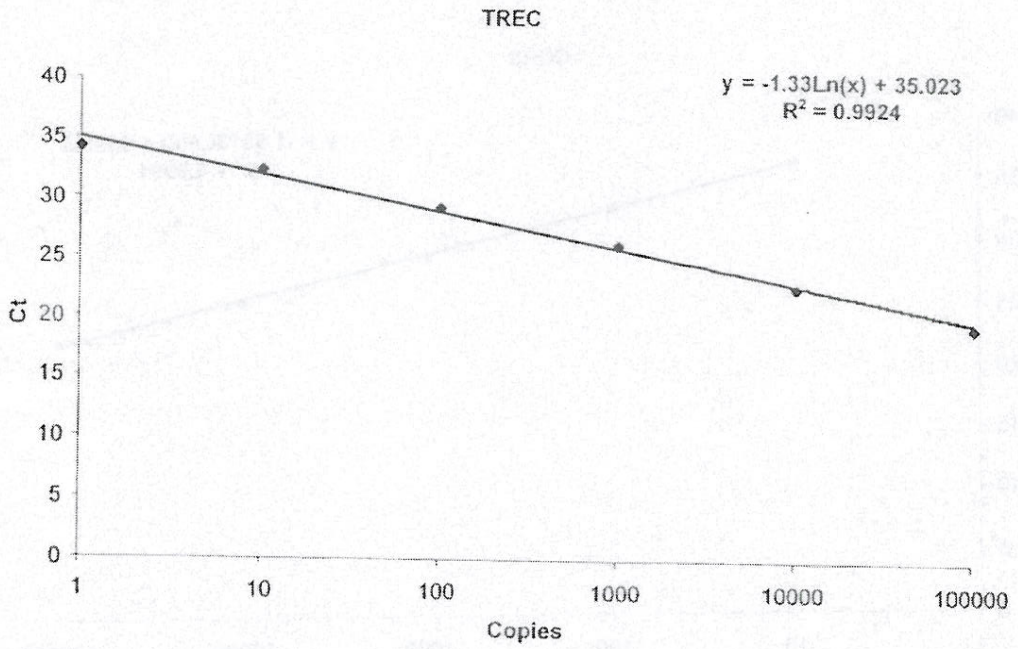

$y = -1.33\text{Ln}(x) + 35.023$
$R^2 = 0.9924$

-Calculate the equation describing the curve. Excel will do this.

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-Use above equations to calculate copies of TRECs and CCR5 in each patient sample.

Patient l CCR5 Ct=27.477 y=-l.5313Ln(x) +39.824
27.477=-1.5313Ln(x) +39.824
x=3175.1 copies of CCR5

Patient 1 TREC Ct=33.322 y=-1.33Ln(x)+35.023
33.322=-1.33Ln(x) + 35.023
x=3.6 TRECs

-Each PBL (diploid cell) contains 2 copies of CCR5. Calculate the number of TRECs/PBL.

Patient 1 has $2.3x10^{-3}$ TRECS/PBL or l TREC/435 PBLs

-When comparing samples between patients, it is important to compare only samples that contain nearly identical CCR5 copy numbers. This is why input DNA is titrated from 0ng-400ng for each patient sample.

-Some useful facts:

1 diploid genome=6.6pg
1ug DNA=151,515 cells
1bp=660Da
1Da=1g/mol
1mole=$6.02x10^{23}$
1bp=$1.09x10^{-21}$g
e=2.718