

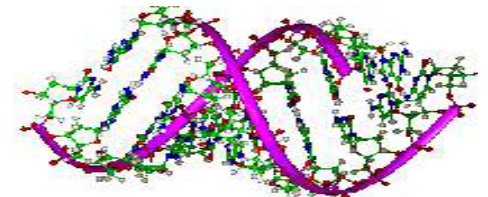
A Sensitive Method for the Quantitation of Residual DNA Using Alu Based Sequences and Real-Time PCR Amplification

Ofer Nussbaum PhD

1st International qPCR Symposium

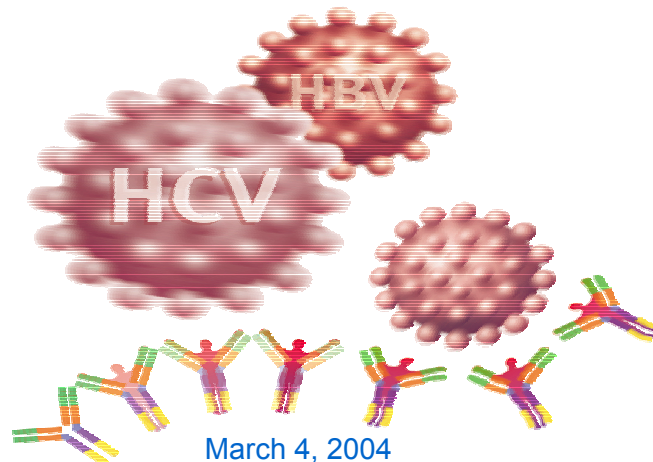
Freising-Weihenstephan, Germany

March, 2004



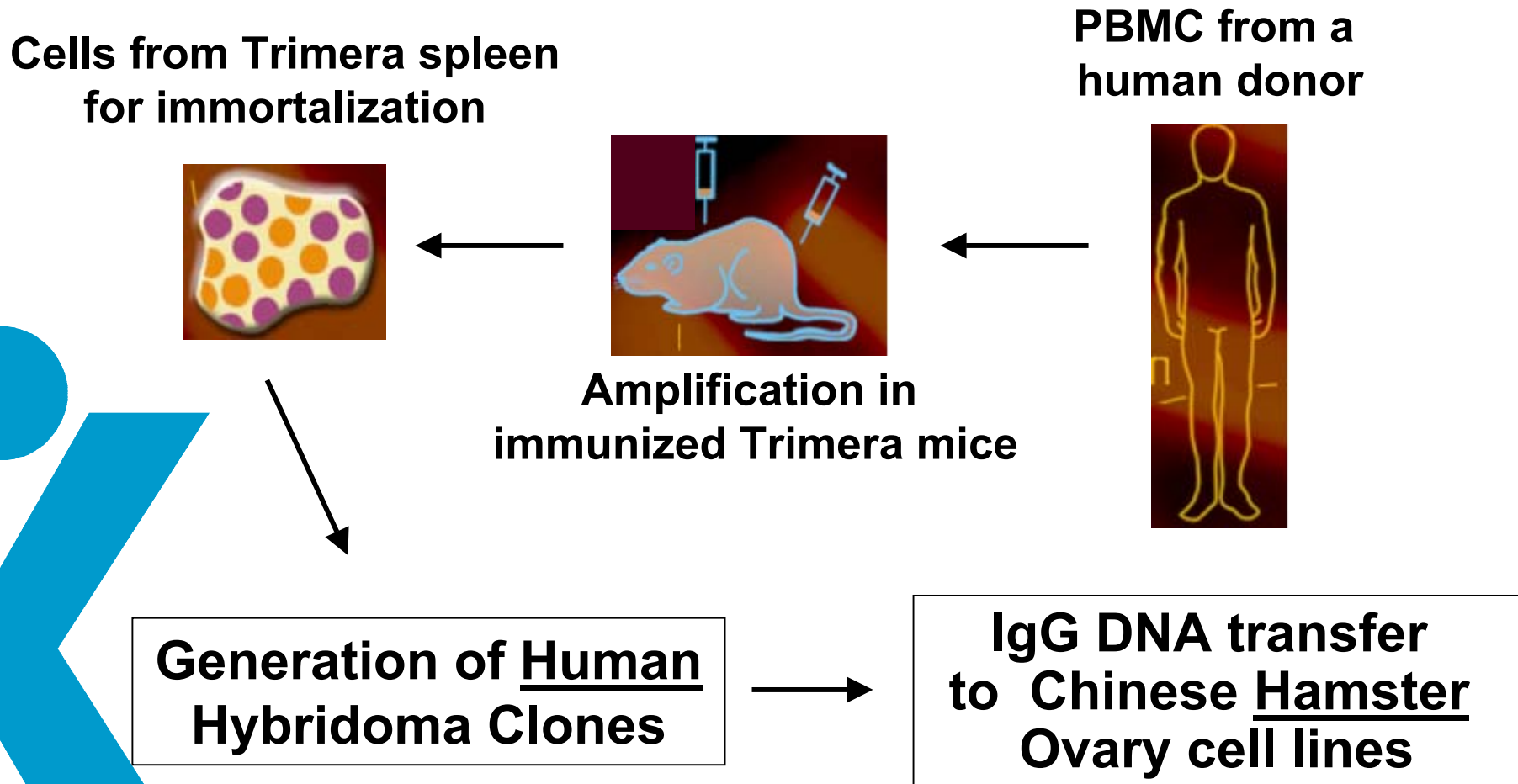
XTL is Developing Human Monoclonal Antibodies to HBV and HCV

These Mab`s are aimed to prevent the re-infection of the transplanted liver in transplanted HBV and HCV patients.



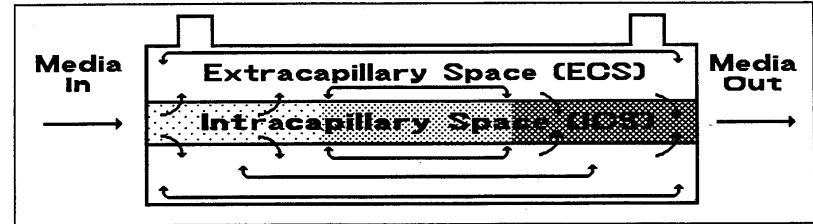
The Trimer Mouse System as a Tool for Generation of Human Monoclonal Antibodies

A Rodent Radiation Chimera with Functioning Human Cells

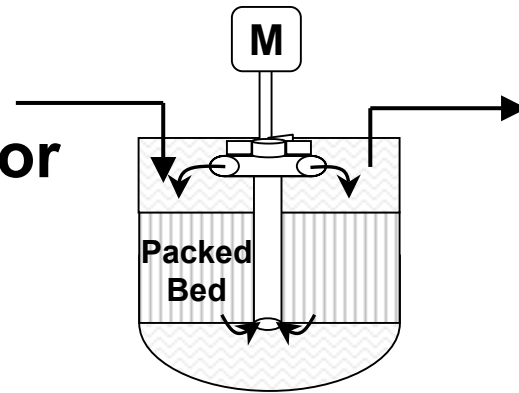


High Scale Production of Monoclonal Antibodies

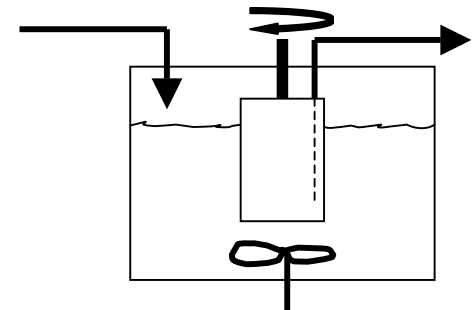
● Hollow Fiber System



● Packed Bed Bioreactor



● Stirred Tank System



Major Steps in Purification of Abs from Cultured Cells Supernatant

- **Protein A column - Abs Isolation and concentration**
- **Anion Exchange column - Removal of residual DNA**
- **DV20F membrane – Virus removal**

Safety Requirements for Clinical Administration of Abs

- **Purity - >95% (SDS-PAGE)**
- **Endotoxin - < 5 EU/ml**
- **Sterility - No Bacteria, Mycoplasma, Viruses**
- **Residual DNA - < 100 pg/dose**



Objective: Development of a highly sensitive method for quantitation of residual genomic DNA in cell cultures supernatants and in purified antibodies.

Current Assays for Estimating Low Levels of Genomic DNA

Quantiblot® Human DNA Quantitation Kit (Applied Biosystems)
Range from 150pg to 10ng of human or primate DNA

AluQuant™ Human DNA Quantitation System (Promega)
Range from 100pg to 50ng of human DNA

Current in-house genomic DNA Quantitation System
Range from 5pg to 500pg of genomic DNA

Strategy: Development of A Quantitative Assay Based on Alu Repeated Sequences and Real Time PCR

- **Primers and probes correlated to the cells spices.**
- **Assay range: 6 logs, from 500fg to 500ng of g-DNA.**
- **Assay should be accurate and reliable.**
- **Assay should be suitable for clinical standards.**

Human Alu: RTF-PCR Primers and Probes

HSU14568 (119 bp) : Human Alu-Sb subfamily consensus.
100% identity over 250 times in the human genome database

5'-GGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGG
AGGCCGAGGCGGGCGGATCACGAGGTCAGGAGATCGAGAC
CATCCTGGCTAACACGGTGAAACCCCGTCTCTACTAAAAA-3'.

Primers and Probe:

gDNAr-F1: 5` - CCTGTAATCCCAGCACTTTGG - 3` 21N

gDNAr-P1: 5` - FAM- AGGCCGGGCGGATC - NFQ -MGB - 3` 13N

gDNAr-R1: 5` - TAGCCAGGATGGTCTCGATCTC - 3` 22N

CHO Alu: RTF-PCR Primers and Probes

pNB137 (J.E. Arrand, 135 bp): Hamster DNA Alu-sequence which is being used to detect transfected hamster DNA.

5` - ATTCCAGCACTCAGGAGGGCAAAGGGCAGAGGATTTCTGAGTTCGAG
 ACCAGCCTGGTCTACAAGAGCTAGTTCCAGGGCAGCCTCCAAAGCCG
 CAGAGAAAAAAGAGGGGTACATCTTGTTGCACAGGAGGGGTCTGTGG - 3`

Primers and Probe:

CHOALU-F1: 5` - GGCAGAGGATTTCTGAGTTCGA- 3` 22N

CHOALU-P: 5` - FAM- ACCAGCCTGGTCTAC - NFQ -MGB - 3` 15N

CHOALU-R1 5` - GGCTGCCCTGGAAGTAGCT- 3` 19N

Purification and Concentration of gDNA from IgG Samples

DNA Extraction (DNAzol- BD methods; MRC, OH, USA)

- **330 μ l sample (1 mg/ml h-IgG)**
- **660 μ l DNAzol-BD**
- **2 μ l glycoblue**
- **500 μ l Isopropanol**

- **Incubate over night at 4⁰C**
- **Wash X1 with 300 μ l DNAzol-BD**
- **Wash X1 with 1 ml 75% Et-OH**
- **Dry and resuspend in 10 μ l of H₂O**

Real Time PCR Protocol

<u>Stock Reagent</u>	<u>1X PCR mix</u>
Taq.-Pol (Promega)	2.5 U
MgCl ₂ (25 mM)	5.0 mM
Betaine 5M	1.0 M
Primer F (50 pm\μl)	50 pmol
Primer R (50 pm\μl)	50 pmol
Probe P (12 pm\μl)	12 pmol
T6-Rox (20X) (12 nmoles\ml)	600 nM
dNTP (10 mM each)	0.2 mM each
PCR buffer (10X, Promega)	1.0 X
H ₂ O	As needed

10 μl DNA sample + 40 μl PCR mix

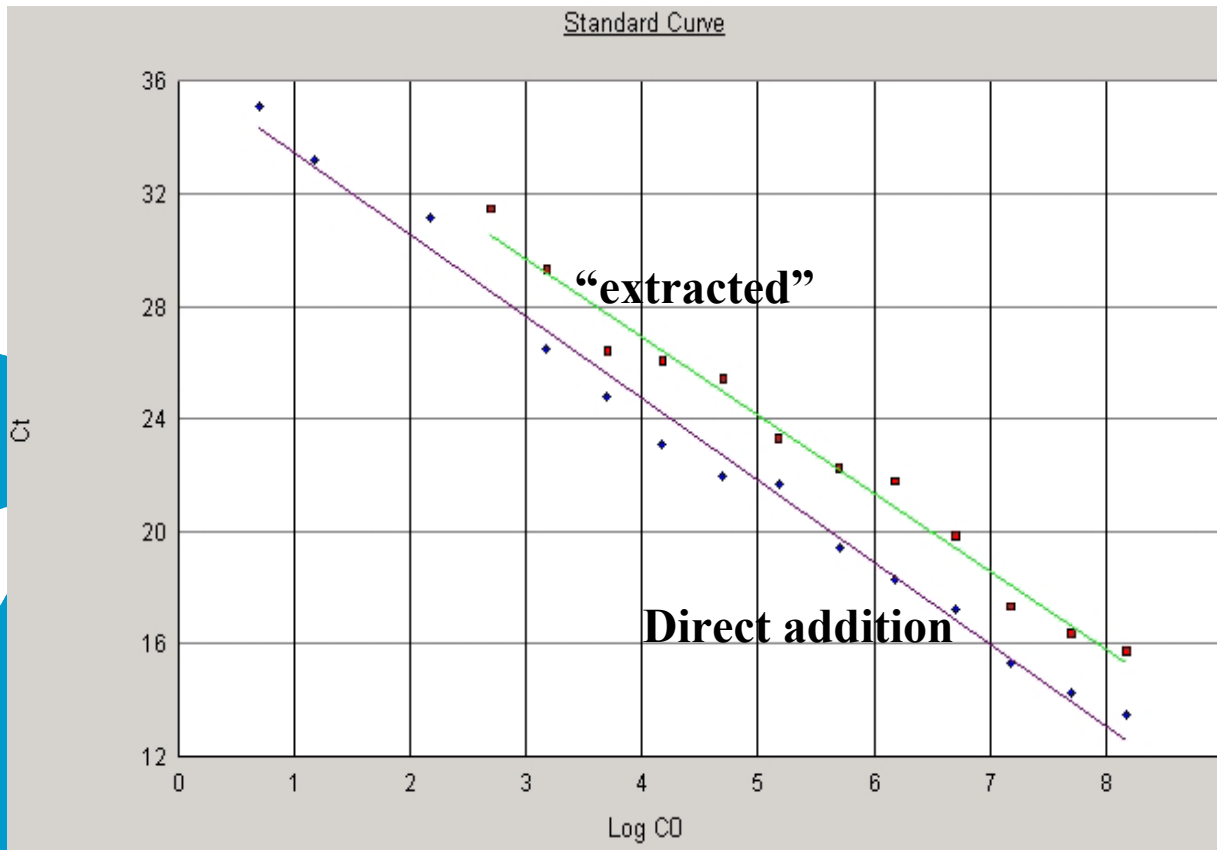
PCR program: Step #1 - 5 min 95°C

ABI-7000
ABI-5700

Step #2 - 40 cycles of:
30 sec. 95°C
60 sec. 60°C

Standard Curves: Direct Addition of DNA Vs. "Extracted" DNA

Stock DNA was extracted from appropriate cultured cells



"extracted" DNA

Serial Dilution
1:2 in 330 μ l PBS/BSA
 $S = -2.8$
 $In. = 37.7$ $R = 0.982$

Directly added DNA

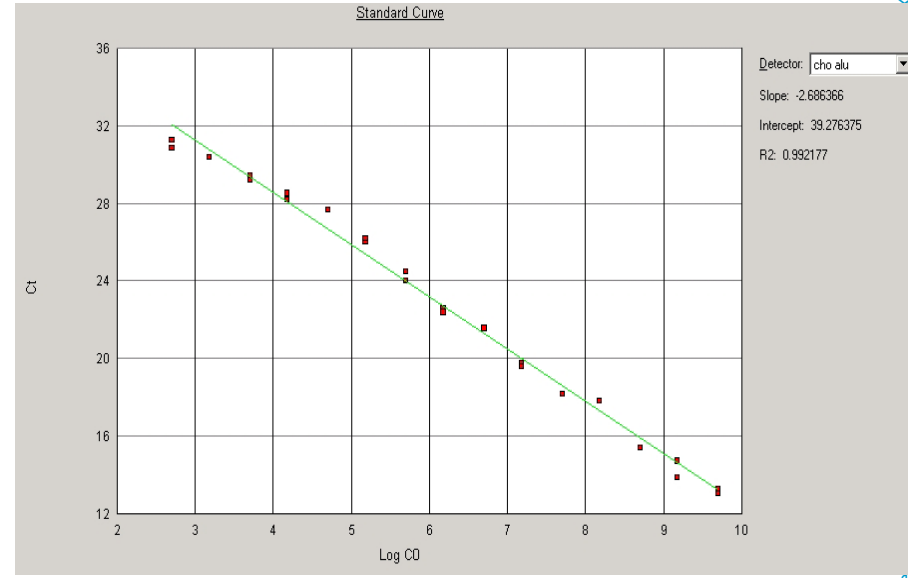
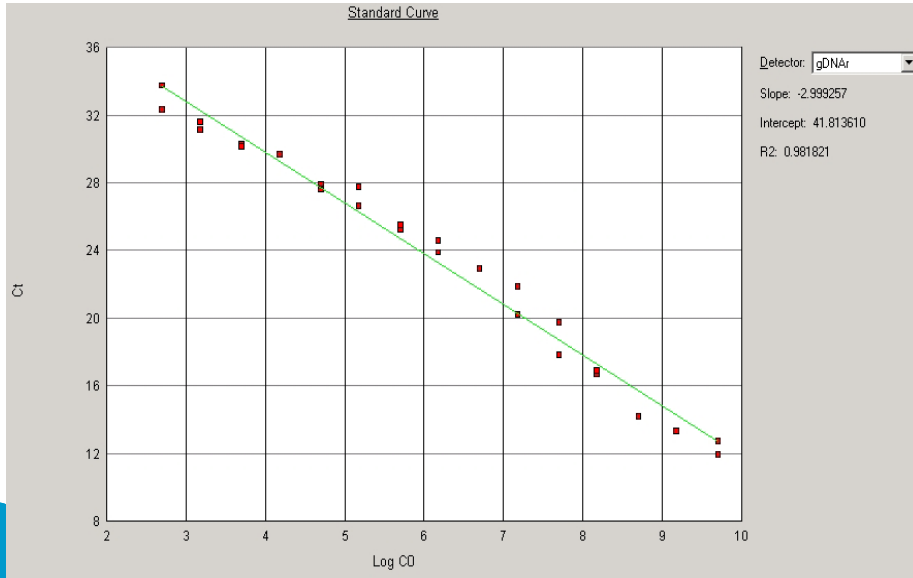
Serial Dilution
1:2 in 10 μ l H₂O
 $S = -2.9$
 $In. = 36.4$ $R = 0.990$

Standard curve and unknown samples were processed in parallel

Standard Curves Range

h DNA - curves

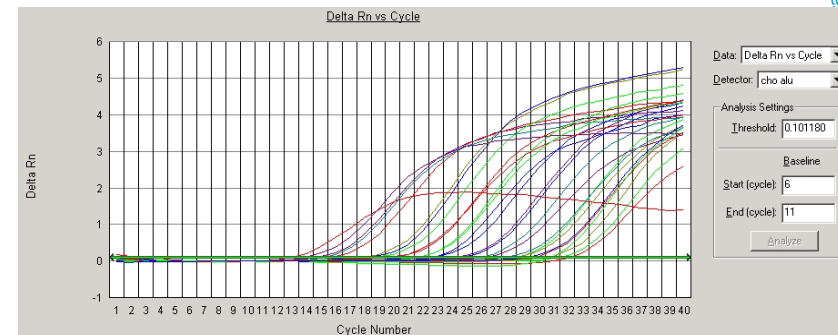
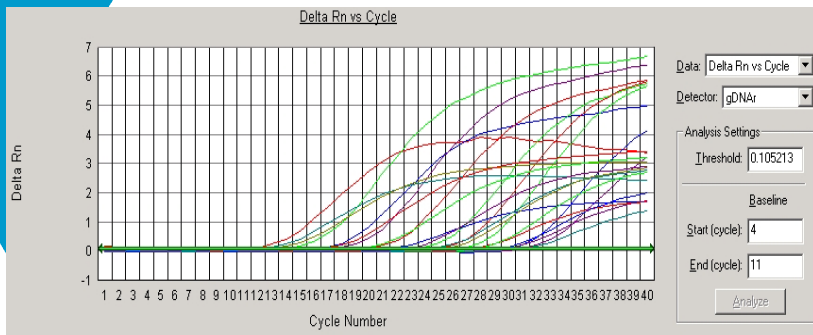
CHO DNA - curves



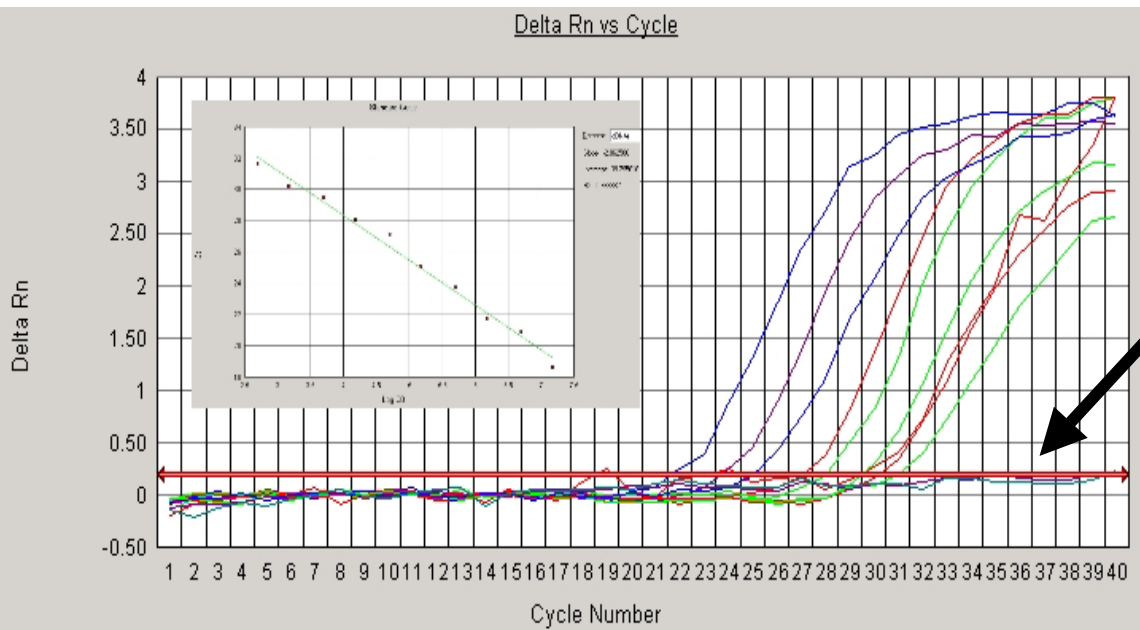
**1.5×10^2 fg/ml
(50 fg DNA)**



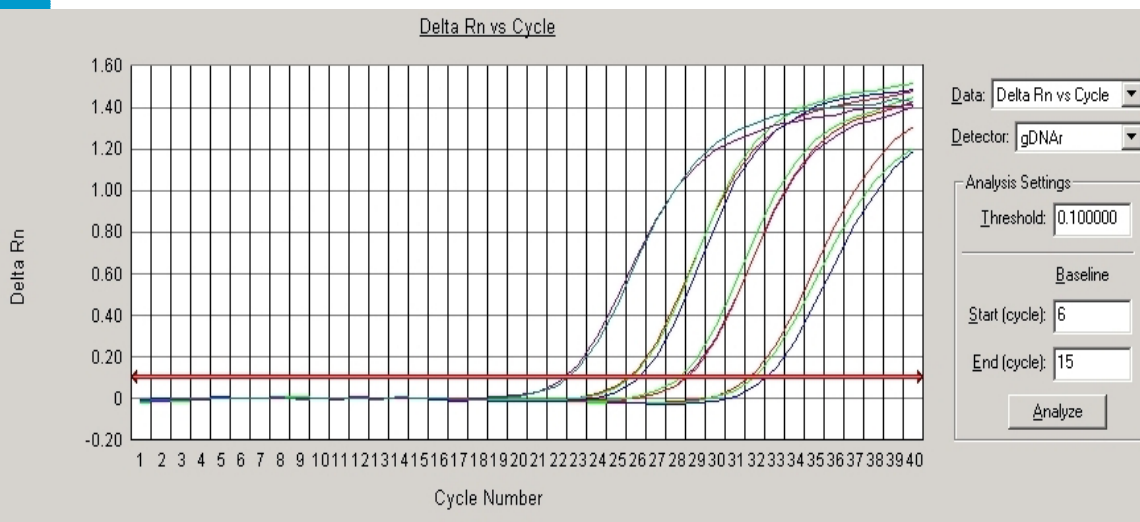
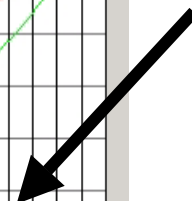
**5×10^9 fg/ml
(1.5 μ g DNA)**



Specificity and Accuracy of the RTF Assay



The human primers and probe failed to recognize the CHO genome (5×10^7 fg/ml)



Un-known samples in triplicates

Assay Sensitivity

There are about 5 pg of DNA in one cell

Alu sequences at about 250 copies in the genome



50 copies / 1 pg DNA



5 copies / 100 fg DNA

Residual DNA detected in Purified Samples

h-Hybridoma cells

DNA – pg/mg IgG

1.0x10⁷

22

1.3

0.3

Purification chart

Supernatant

Protein A column

Anion Exchange column

DV20F membrane

CHO cells

DNA – pg/mg IgG

2.25x10⁷

36

2.2

0.6

Purified Antibodies contain 0.3-0.6 pg DNA / mg IgG

Conclusions

- **We have developed an assay for the determination of residual DNA in purified monoclonal antibody preparations and in a supernatant of cultured cells.**
- **The assay is based on Real Time PCR techniques employing primers and Taq-man MGB-probe correlating to Alu like sequences, using DNazol for DNA extraction and concentration.**
- **The assay is species specific, accurate and has a wide range of 8 logs between 50 fg to 5 μ g of genomic DNA.**
- **Using this assay we have determined the residual DNA levels in clinical material administered to patients, in our HBV and HCV clinical trials.**

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