

Biosearch Technologies, Inc.



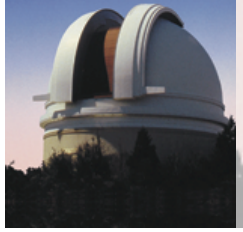
Chemistry for Genomics and Proteomics℠

www.biosearchtech.com
info@biosearchtech.com

FROM SEQUENCES TO SYNTHESIS:

Optimal Amplification through Careful Oligonucleotide Selection

Ben Sowers, Research Associate
ben@biosearchtech.com



Multiplexing requires matching reporters to instrument optics

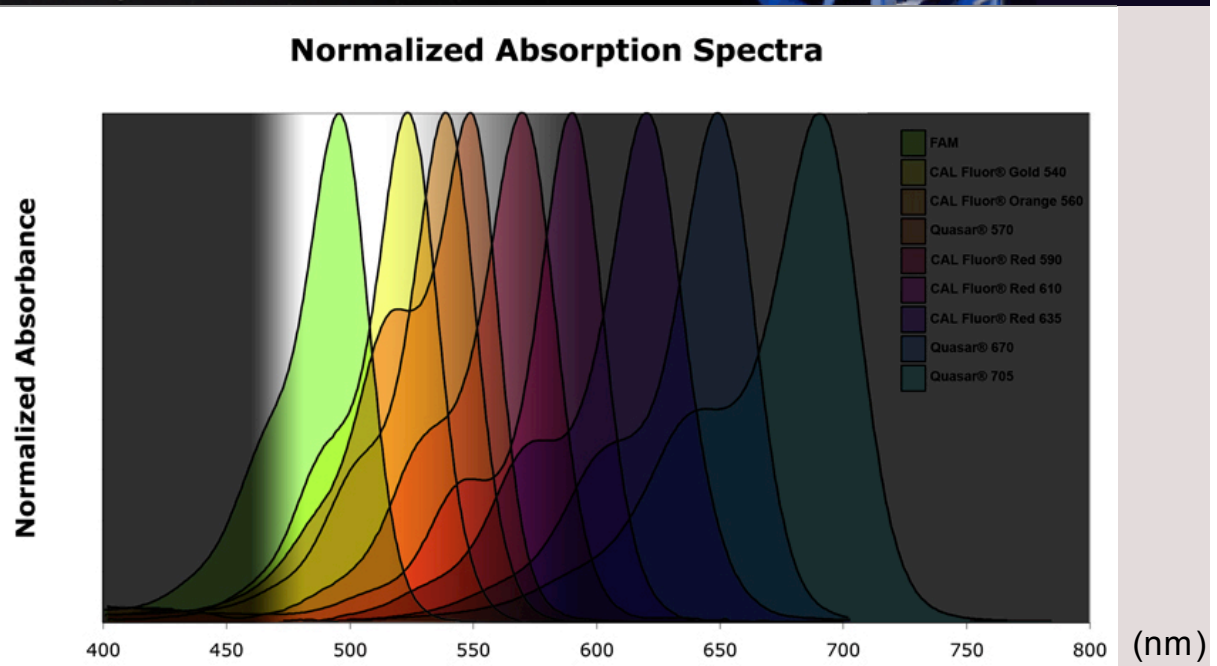
Factors to Consider:

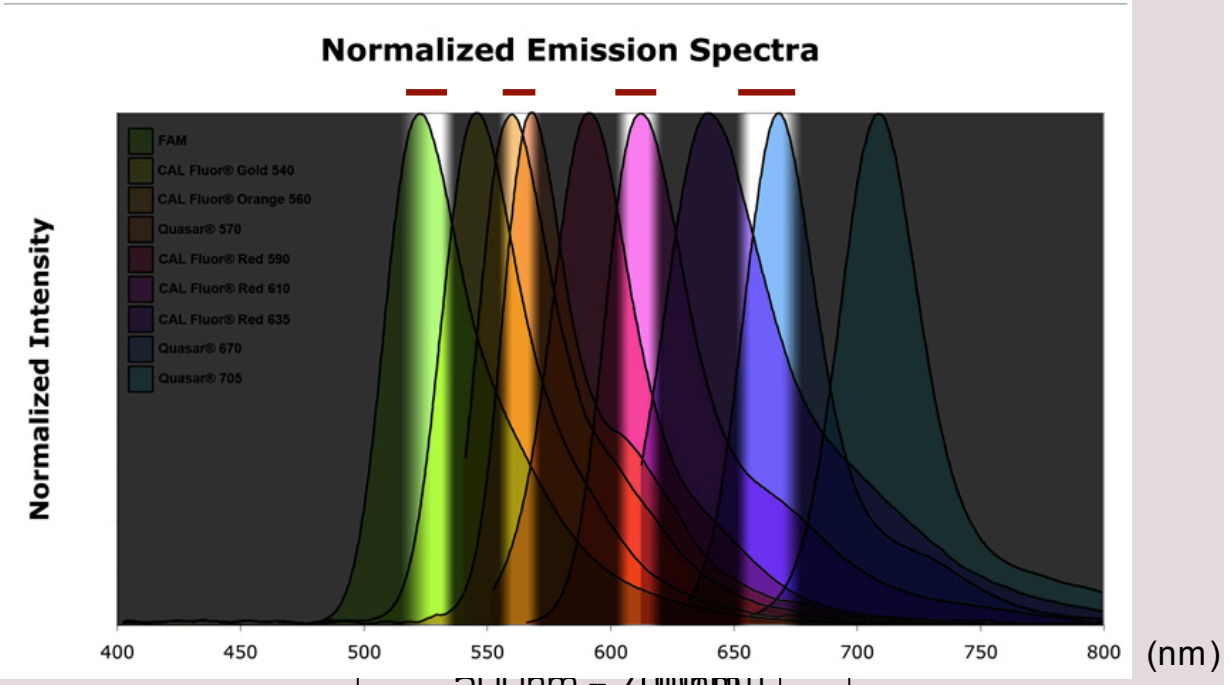
1) <u>spectral overlap</u>
   can signals be resolved from one another?

2) <u>emission filters</u>
   do the emission spectra align with the filters?

3) <u>excitation filters</u>
   do the absorption spectra align with the filters?

4) <u>excitation source</u>
   does the lamp/laser/LED excite the dyes?





Multiplexing requires matching reporters to instrument optics

Multiplexing Recommendations[1] for Dual-labeled Dark-quenched Probes and Primers

INSTRUMENT	MANF.	CALIBRATION REQ'D?	REC. DEGREE OF MULTIPLEXING	DYE 1	DYE 2	DYE 3	DYE 4	DYE 5
LightCycler 2.0	Roche	Yes[4]	Triplex	FAM	CAL Fluor Red 610	Pulsar 650		

Determined through research at Biosearch

Determined through research of a collaborator

Predicted, based on instrument specifications

1 These recommendations apply to dual-labeled BHQ-quenched probes (TaqMan® Probes, Molecular Beacons) and BHQ-quenched primers (Black Hole Scorpions, Amplifluor Direct, etc.). They should not be used as guidelines for TAMRA-quenched probes or for Hybridization Probes that rely on FRET as a means of excitation. Recommendations highlighted in blue have not been proven and so should be used cautiously on an experimental basis. Stratagene customers select their filters from among a series upon instrument purchase. Because multiplexing dye compatibility is affected by filter specifications, the above Stratagene recommendations only apply to the standard FAM/HEX/ROX/Cy5 filter set.

2 Some instruments will require fluorescence calibration. Fluorescence calibration allows these instruments to record the spectral profile for the dye(s) to be used in subsequent assays by resolving the total detected light into signals contributed by the individual fluorophores. Please visit www.biosearchtech.com for information on our calibration dyes.

3 SuperRox is a proprietary passive reference available from Biosearch.

4 LightCycler users can calibrate using TaqMan® probes directly and therefore aren't required to purchase dye calibration kits to calibrate their instrument.

Instrument Compatibility for Dyes Available Through Biosearch[1]

INSTRUMENT	MANUFACTURER	CALIBRATION REQUIRED?[2]	FAM™	TET™	CAL FLUOR® GOLD 540	HEX™	CAL FLUOR ORANGE 560	QUASAR® 570	TAMRA™	CAL FLUOR RED 590	CAL FLUOR RED 610	CAL FLUOR RED 635	PULSAR® 650	QUASAR 670
Prism® 7700	ABI	Yes	■	■	■	■	■	■	■	■	■	■	■	■
Prism 7900	ABI	Yes	■	■	■	■	■	■	■	■	■	■	■	■
Prism 7000	ABI	Yes	■	■	■	■	■	■	■	■	■	■	■	■
Prism 7300	ABI	Yes	■	■	■	■	■	■	■	■	■	■	■	■
Prism 7500	ABI	Yes	■	■	■	■	■	■	■	■	■	■	■	■
iCycler®	Bio-Rad	Yes	■	■	■	■	■	■	■	■	■	■	■	■
SmartCycler®	Cepheid	Yes	■	■	■	■	■	■	■	■	■	■	■	■
SmartCycler II	Cepheid	Yes	■	■	■	■	■	■	■	■	■	■	■	■
Opticon® 2	MJ Research/Bio-Rad	Yes	■	■	■	■	■	■	■	■	■	■	■	■
Chromo™ 4	MJ Research/Bio-Rad	Yes	■	■	■	■	■	■	■	■	■	■	■	■
Rotor-Gene™ 3000	Corbett Research	No	■	■	■	■	■	■	■	■	■	■	■	■
MX3000P™	Stratagene	No	■	■	■	■	■	■	■	■	■	■	■	■
MX4000®	Stratagene	No	■	■	■	■	■	■	■	■	■	■	■	■
LightCycler® 1.2	Roche	Yes[3]	■	■	■	■	■	■	■	■	■	■	■	■
LightCycler 2.0	Roche	Yes[3]	■	■	■	■	■	■	■	■	■	■	■	■

1 This chart indicates the instrument specific compatibility of dyes when used as reporters in dual-labeled, dark-quenched probes. This chart only describes compatibility for singleplex reactions and does not suggest the best choices for multiplexing. For multiplexing recommendations please see the "Multiplexing Recommendations" chart.

2 Some instruments will require fluorescence calibration. Fluorescence calibration allows these instruments to record the spectral profile for the dye(s) to be used in subsequent assay by resolving the total detected light into signals contributed by the individual fluorophores. Please visit www.biosearchtech.com for information on our calibration dyes.

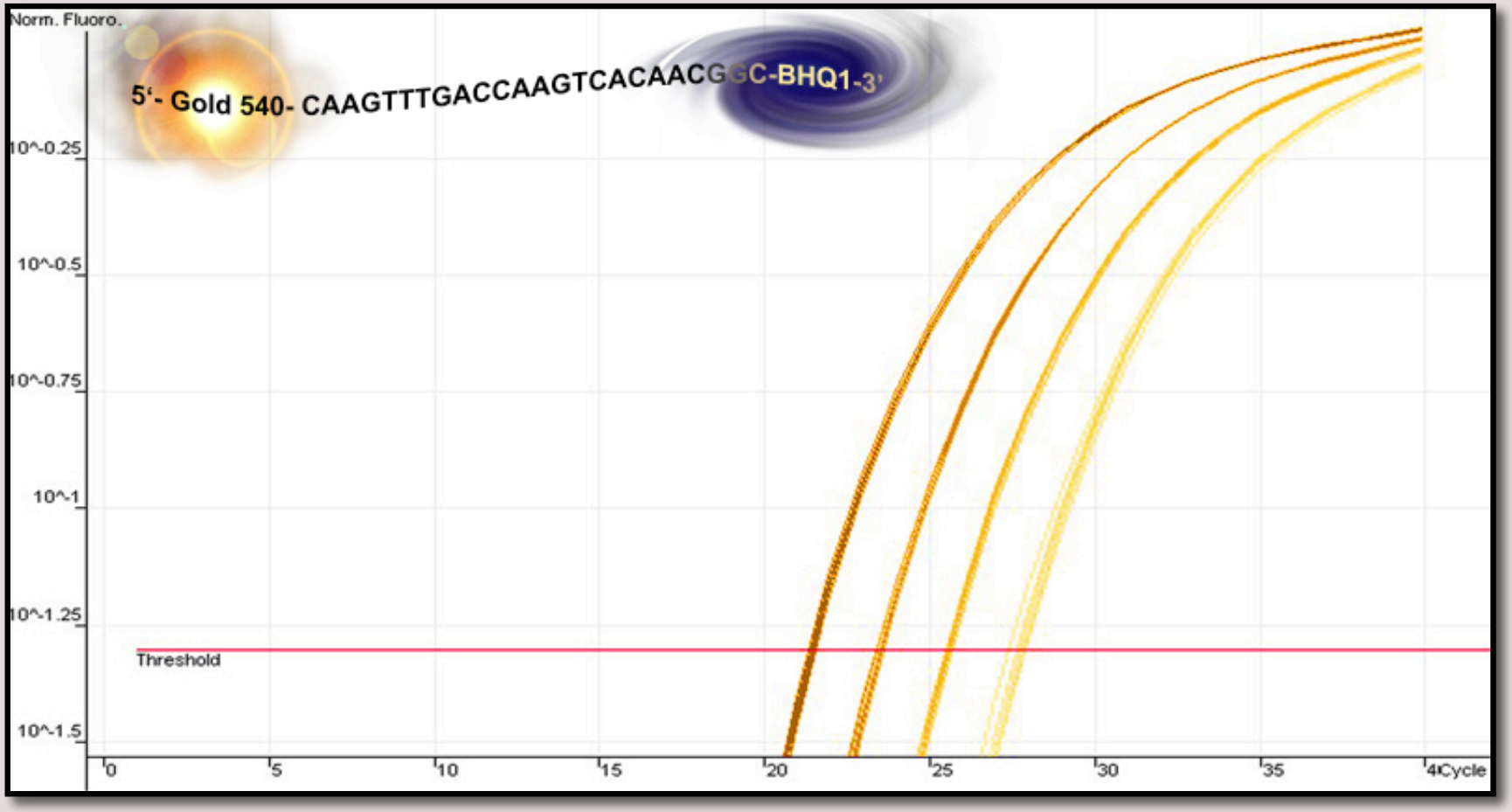
3 LightCycler users can calibrate using TaqMan® probes directly and therefore aren't required to purchase dye calibration kits to calibrate their instrument.

■ COMPATIBLE *(Either described as compatible in instrument documentation or proven compatible through internal or collaborator research)*

■ INCOMPATIBLE *(Either described as incompatible in instrument documentation or proven incompatible through internal or collaborator research)*

■ UNTESTED BUT PRESUMED TO BE COMPATIBLE

www.multiplexqpcr.com


Norm. Fluoro.
5'- Gold 540- CAAGTTTGACCAAGTCACAACGGC-BHQ1-3'
10^-0.25
10^-0.5
10^-0.75
10^-1
10^-1.25
10^-1.5
Threshold
0
5
10
15
20
25
30
35
40
Cycle

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REALTIMEDESIGN software

An advanced web-based program for real-time PCR Sequence Design

www.biosearchtech.com/realtimedesign



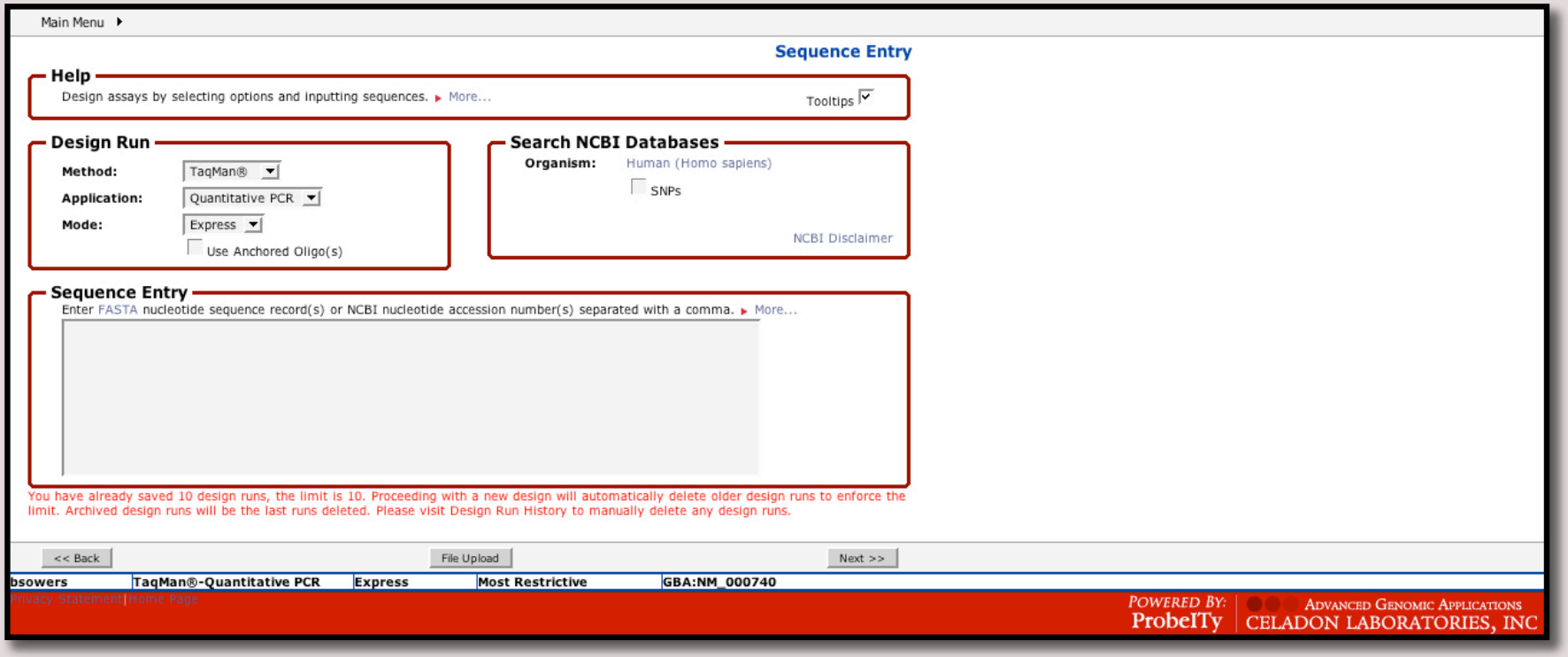
Summary of Features:

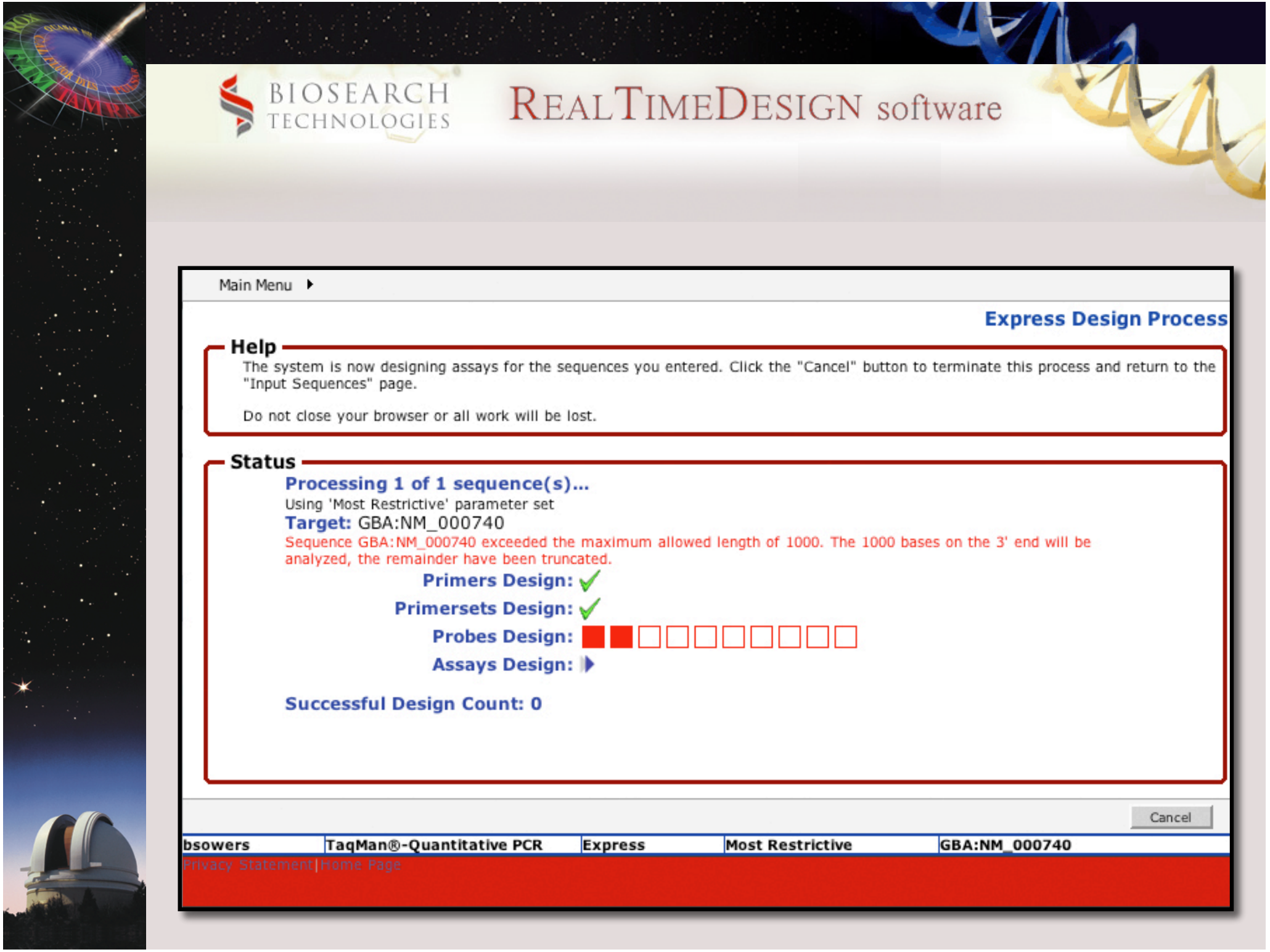
1) The default parameters are fine-tuned so that no further adjustment is required by the user
2) Interfacing with NCBI's databases facilitates sequence retrieval using accession numbers, as well as BLAST searches for SNP identification
3) Electronic PCR (ePCR) submission allows immediate confirmation of specificity
4) Comprehensive parameters are user-adjustable to overcome difficult targets
5) Up to 10 different targets can be submitted and designed simultaneously, the results of which are archived for viewing later

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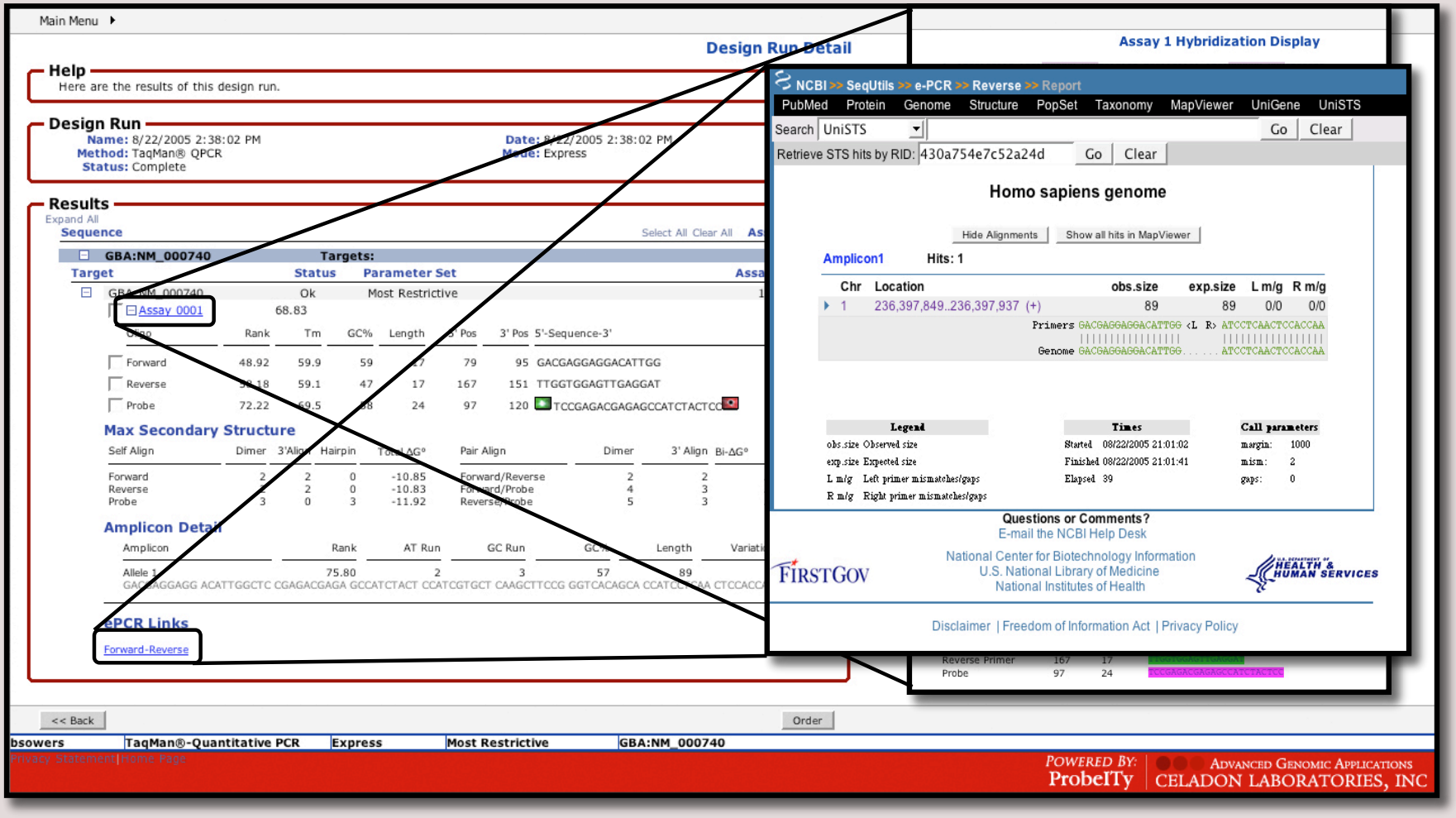
REALTIMEDESIGN software

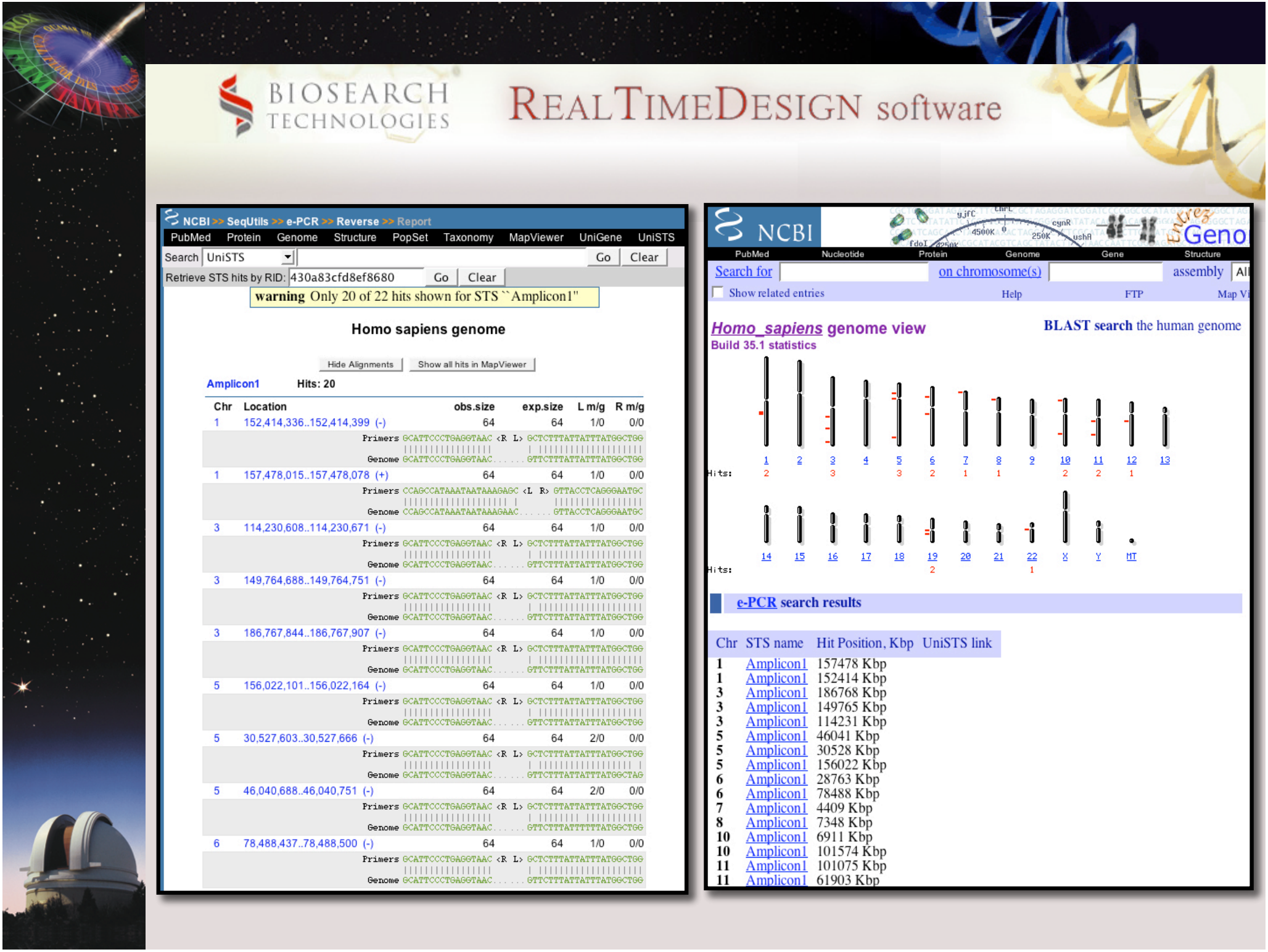


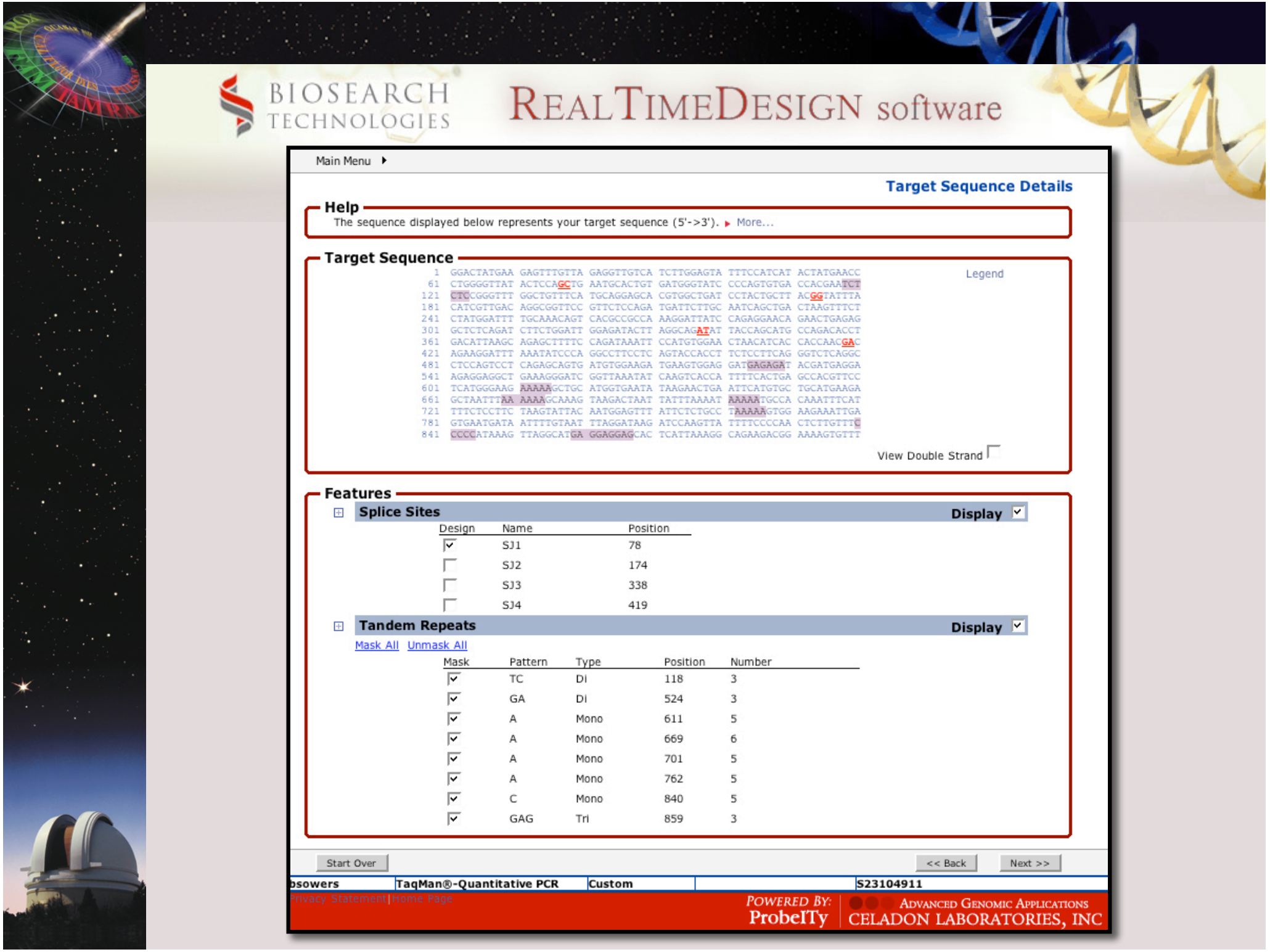

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REALTIMEDESIGN software
Main Menu
Express Design Process
Help
The system is now designing assays for the sequences you entered. Click the "Cancel" button to terminate this process and return to the "Input Sequences" page.
Do not close your browser or all work will be lost.
Status
Processing 1 of 1 sequence(s)...
Using 'Most Restrictive' parameter set
Target: GBA:NM_000740
Sequence GBA:NM_000740 exceeded the maximum allowed length of 1000. The 1000 bases on the 3' end will be analyzed, the remainder have been truncated.
Primers Design:
Primersets Design:
Probes Design:
Assays Design:
Successful Design Count: 0
Cancel
bsowers
TaqMan®-Quantitative PCR
Express
Most Restrictive
GBA:NM_000740
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NCBI >> SeqUtils >> e-PCR >> Reverse >> Report
PubMed Protein Genome Structure PopSet Taxonomy MapViewer UniGene UniSTS
Search UniSTS Go Clear
Retrieve STS hits by RID: 430a83cfd8ef8680 Go Clear
warning Only 20 of 22 hits shown for STS ``Amplicon1"
Homo sapiens genome
Hide Alignments Show all hits in MapViewer
Amplicon1 Hits: 20
Chr Location obs.size exp.size L m/g R m/g
1 152,414,336..152,414,399 (-) 64 64 1/0 0/0
Primers GCATTCCCTGAGGTAAC <R L> GCTCTTTATTATTTATGGCTGG
Genome GCATTCCCTGAGGTAAC..... GTTCTTTATTATTTATGGCTGG
1 157,478,015..157,478,078 (+) 64 64 1/0 0/0
Primers CCAGCCATAAATAATAAAGAGC <L R> GTTACCTCAGGGAATGC
Genome CCAGCCATAAATAATAAAGAAC..... GTTACCTCAGGGAATGC
3 114,230,608..114,230,671 (-) 64 64 1/0 0/0
Primers GCATTCCCTGAGGTAAC <R L> GCTCTTTATTATTTATGGCTGG
Genome GCATTCCCTGAGGTAAC..... GTTCTTTATTATTTATGGCTGG
3 149,764,688..149,764,751 (-) 64 64 1/0 0/0
Primers GCATTCCCTGAGGTAAC <R L> GCTCTTTATTATTTATGGCTGG
Genome GCATTCCCTGAGGTAAC..... GTTCTTTATTATTTATGGCTGG
3 186,767,844..186,767,907 (-) 64 64 1/0 0/0
Primers GCATTCCCTGAGGTAAC <R L> GCTCTTTATTATTTATGGCTGG
Genome GCATTCCCTGAGGTAAC..... GTTCTTTATTATTTATGGCTGG
5 156,022,101..156,022,164 (-) 64 64 1/0 0/0
Primers GCATTCCCTGAGGTAAC <R L> GCTCTTTATTATTTATGGCTGG
Genome GCATTCCCTGAGGTAAC..... GTTCTTTATTATTTATGGCTGG
5 30,527,603..30,527,666 (-) 64 64 2/0 0/0
Primers GCATTCCCTGAGGTAAC <R L> GCTCTTTATTATTTATGGCTGG
Genome GCATTCCCTGAGGTAAC..... GTTCTTTATTATTTATGGCTAG
5 46,040,688..46,040,751 (-) 64 64 2/0 0/0
Primers GCATTCCCTGAGGTAAC <R L> GCTCTTTATTATTTATGGCTGG
Genome GCATTCCCTGAGGTAAC..... GTTCTTTATTTTTTATGGCTGG
6 78,488,437..78,488,500 (-) 64 64 1/0 0/0
Primers GCATTCCCTGAGGTAAC <R L> GCTCTTTATTATTTATGGCTGG
Genome GCATTCCCTGAGGTAAC..... GTTCTTTATTATTTATGGCTGG
NCBI
PubMed Nucleotide Protein Genome Gene Structure
Search for on chromosome(s) assembly
Show related entries Help FTP
Homo_sapiens genome view
Build 35.1 statistics
BLAST search the human genome
Hits:
Hits:
e-PCR search results
Chr STS name Hit Position, Kbp UniSTS link
1 Amplicon1 157478 Kbp
1 Amplicon1 152414 Kbp
3 Amplicon1 186768 Kbp
3 Amplicon1 149765 Kbp
3 Amplicon1 114231 Kbp
5 Amplicon1 46041 Kbp
5 Amplicon1 30528 Kbp
5 Amplicon1 156022 Kbp
6 Amplicon1 28763 Kbp
6 Amplicon1 78488 Kbp
7 Amplicon1 4409 Kbp
8 Amplicon1 7348 Kbp
10 Amplicon1 6911 Kbp
10 Amplicon1 101574 Kbp
11 Amplicon1 101075 Kbp
11 Amplicon1 61903 Kbp


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REALTIMEDESIGN software
Main Menu
Target Sequence Details
Help
The sequence displayed below represents your target sequence (5'->3'). More...
Target Sequence
Legend
1 GGACTATGAA GAGTTTGTTA GAGGTTGTCA TCTTGGAGTA TTTCCATCAT ACTATGAACC
61 CTGGGGTTAT ACTCCAGCTG AATGCACTGT GATGGGTATC CCCAGTGTGA CCACGAATCT
121 CTCCGGGTTT GGCTGTTTCA TGCAGGAGCA CGTGGCTGAT CCTACTGCTT ACGGTATTTA
181 CATCGTTGAC AGGCGGTTCC GTTCTCCAGA TGATTCTTGC AATCAGCTGA CTAAGTTTCT
241 CTATGGATTT TGCAAACAGT CACGCCGCCA AAGGATTATC CAGAGGAACA GAACTGAGAG
301 GCTCTCAGAT CTTCTGGATT GGAGATACTT AGGCAGATAT TACCAGCATG CCAGACACCT
361 GACATTAAGC AGAGCTTTTC CAGATAAATT CCATGTGGAA CTAACATCAC CACCAACGAC
421 AGAAGGATTT AAATATCCCA GGCCTTCCTC AGTACCACCT TCTCCTTCAG GGTCTCAGGC
481 CTCCAGTCCT CAGAGCAGTG ATGTGGAAGA TGAAGTGGAG GATGAGAGAT ACGATGAGGA
541 AGAGGAGGCT GAAAGGGATC GGTTAAATAT CAAGTCACCA TTTTCACTGA GCCACGTTCC
601 TCATGGGAAG AAAAAGCTGC ATGGTGAATA TAAGAACTGA ATTCATGTGC TGCATGAAGA
661 GCTAATTTAA AAAAGCAAAG TAAGACTAAT TATTTAAAAT AAAAATGCCA CAAATTTCAT
721 TTTCTCCTTC TAAGTATTAC AATGGAGTTT ATTCTCTGCC TAAAAAGTGG AAGAAATTGA
781 GTGAATGATA ATTTTGTAAT TTAGGATAAG ATCCAAGTTA TTTTCCCCAA CTCTTGTTTC
841 CCCCATAAAG TTAGGCATGA GGAGGAGCAC TCATTAAAGG CAGAAGACGG AAAAGTGTTT
View Double Strand
Features
Splice Sites
Display
Design Name Position
SJ1 78
SJ2 174
SJ3 338
SJ4 419
Tandem Repeats
Display
Mask All Unmask All
Mask Pattern Type Position Number
TC DI 118 3
GA DI 524 3
A Mono 611 5
A Mono 669 6
A Mono 701 5
A Mono 762 5
C Mono 840 5
GAG Tri 859 3
Start Over
<< Back
Next >>
bsowers
TaqMan®-Quantitative PCR
Custom
S23104911
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CELADON LABORATORIES, INC

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REALTIMEDESIGN software

Main Menu

Select Design Parameters

Help

If desired, click the "View/Edit" link below to modify the default parameters. More...

Parameter Settings

Parameter Set: Most Restrictive | View/Edit

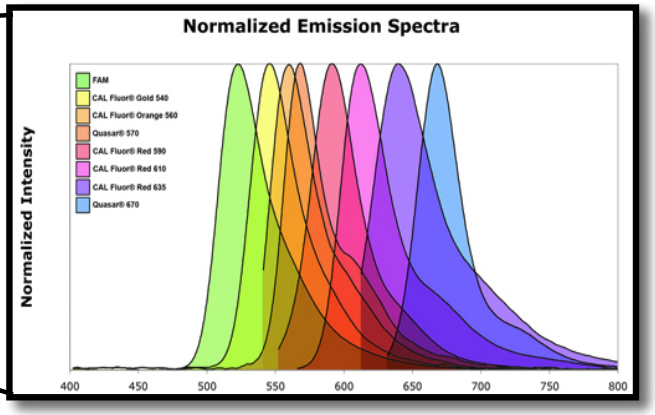
Dual Label Selection

5' Label Type - 3' Label Type: 5'Fluorescent Reporter - 3'Dark Quencher

Label for the TaqMan probe:

CAL Fluor® Gold 540	BHQ-1
CAL Fluor® Orange 560	BHQ-1
CAL Fluor® Red 590	BHQ-2
CAL Fluor® Red 610	BHQ-2
CAL Fluor® Red 635	BHQ-2
FAM	BHQ-1
HEX	BHQ-2
JOE	BHQ-2
Quasar® 570	BHQ-2
Quasar® 670	BHQ-2

Due to differences in real-time PCR thermal cyclers, the choice of fluorescent reporter for a probe should be carefully considered against the instrument's excitation source, filter arrangement, and detection capabilities. If this probe is to be combined with others in a multiplexed assay then the emission spectra of each probe's reporter need to be sufficiently resolved (minimum 20nm) for the instrument to separate the signals from one another. Many real-time instruments require spectral calibration prior to detecting any non-standard reporter dye. Because dye calibration settings cannot be substituted for one another, pure dye calibration solutions must be obtained either from Biosearch Technologies or the instrument manufacturer.



Start Over | << Back | Next >>

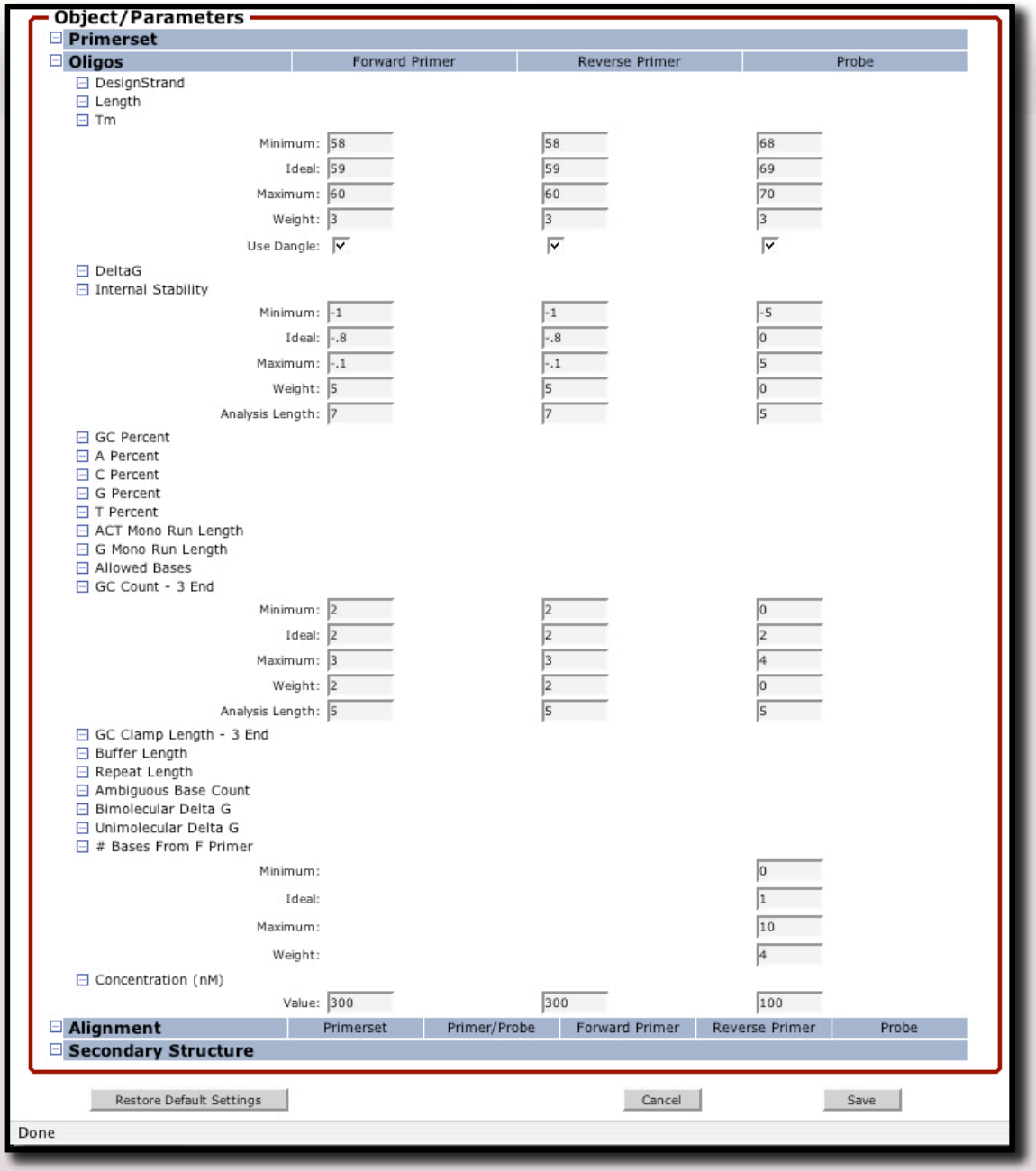
bsowers	TaqMan®-Quantitative PCR	Custom	Most Restrictive	S23104911

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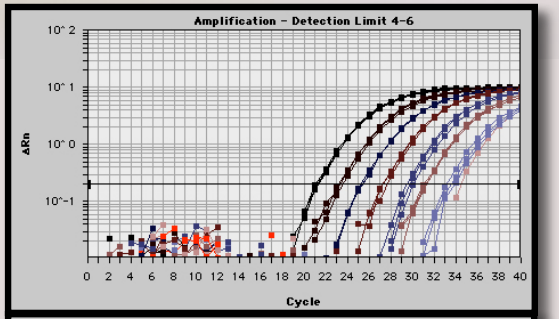
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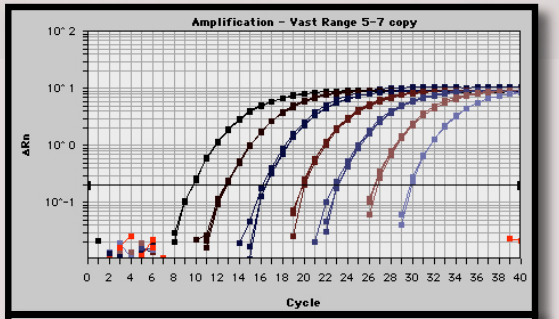
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Human Genomic DNA

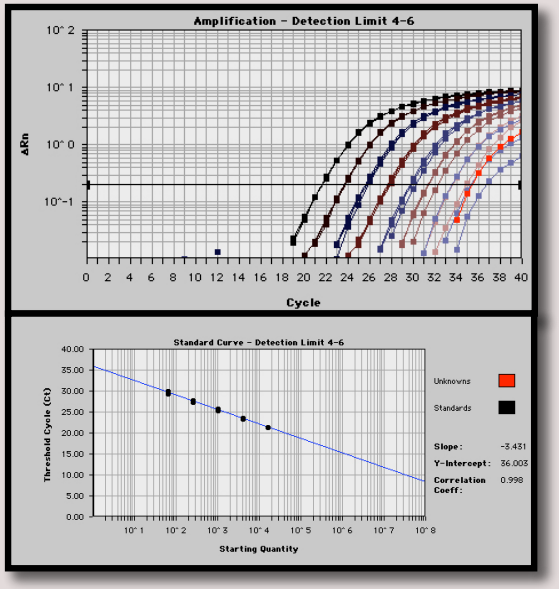


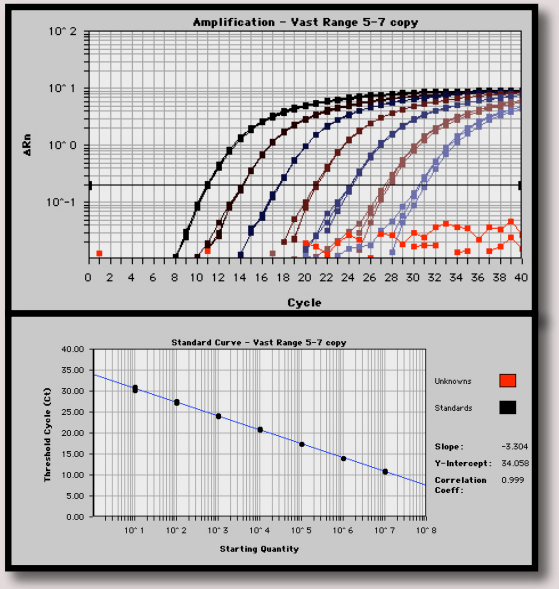
Purified PCR Product



Glycogen synthase 2 (GYS2)

Amp. Efficiency (genomic DNA):	96.5%
R^2 (genomic DNA):	0.997
Assay sensitivity (genomic DNA):	256 copies
Amp. Efficiency (PCR Product):	97.6%
R^2 (PCR Product):	0.999





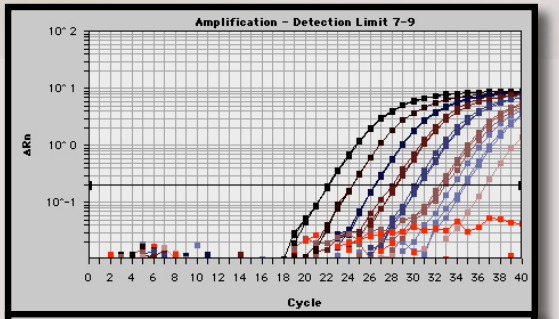
Cholinergic receptor, muscarinic 3 (CHRM3)

Amp. Efficiency (genomic DNA):	95.6%
R^2 (genomic DNA):	0.998
Assay sensitivity (genomic DNA):	64 copies
Amp. Efficiency (PCR Product):	100.8%
R^2 (PCR Product):	0.999

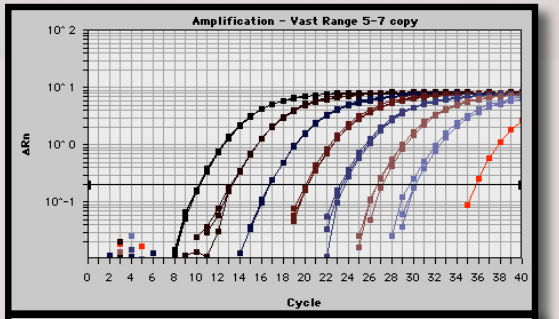
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Human Genomic DNA

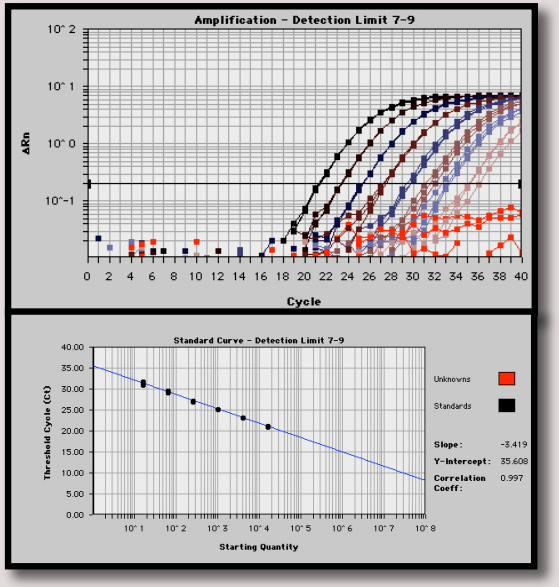


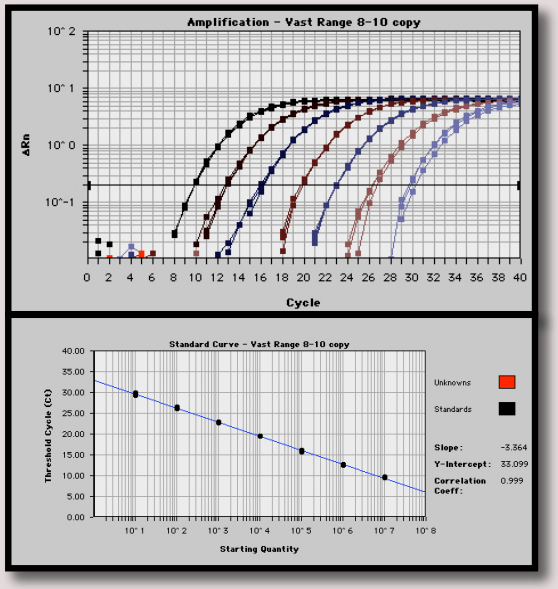
Purified PCR Product



L amino acid transporter-2 (SLC7A8)

Amp. Efficiency (genomic DNA):	95.4%
R^2 (genomic DNA):	0.998
Assay sensitivity (genomic DNA):	16 copies
Amp. Efficiency (PCR Product):	100.1%
R^2 (PCR Product):	0.999





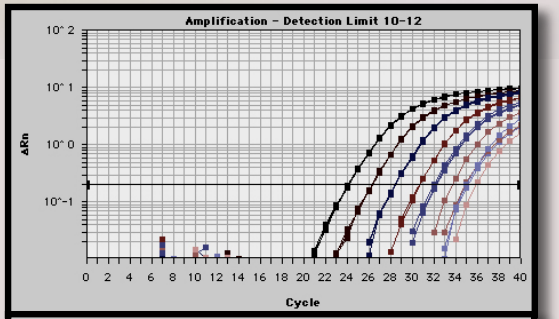
SMAD, mothers agains DPP homolog 4 (SMAD4)

Amp. Efficiency (genomic DNA):	96.1%
R^2 (genomic DNA):	0.997
Assay sensitivity (genomic DNA):	16 copies
Amp. Efficiency (PCR Product):	98.3%
R^2 (PCR Product):	0.999

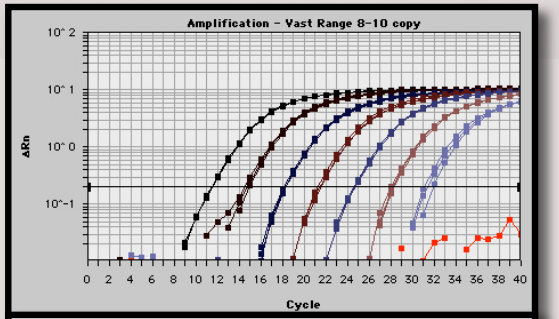
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REALTIMEDESIGN software

Human Genomic DNA

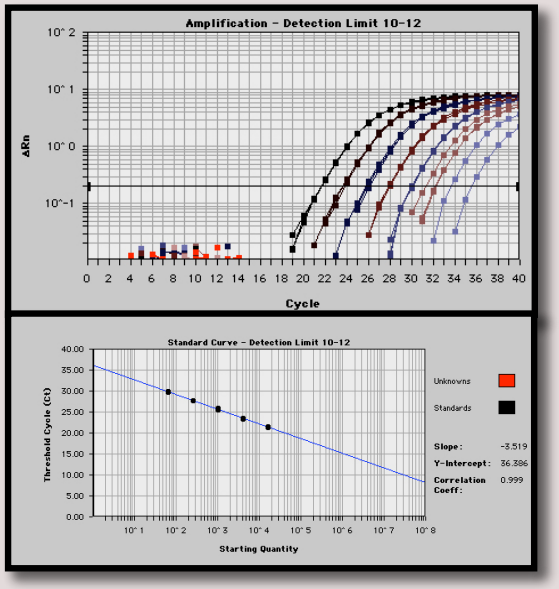


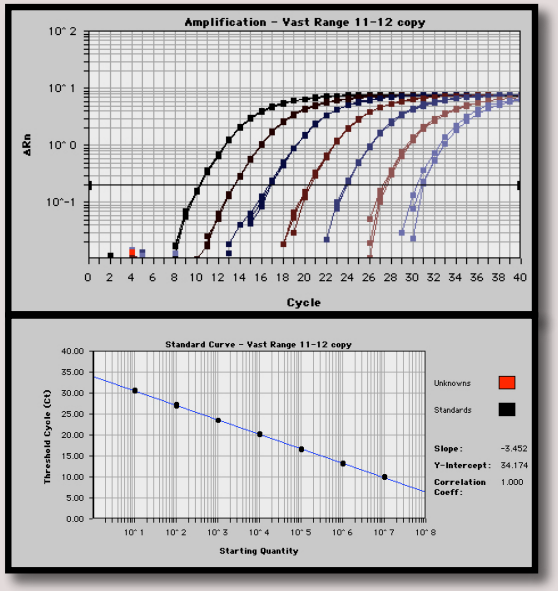
Purified PCR Product



Eukaryotic translation initiation factor 5 (EIF5)

Amp. Efficiency (genomic DNA):	98.0%
R^2 (genomic DNA):	0.997
Assay sensitivity (genomic DNA):	64 copies
Amp. Efficiency (PCR Product):	99.5%
R^2 (PCR Product):	0.999





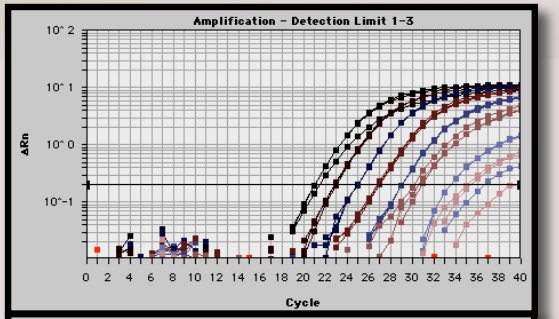
ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C, isoform 1 (ATP6V1C1)

Amp. Efficiency (genomic DNA):	92.3%
R^2 (genomic DNA):	0.999
Assay sensitivity (genomic DNA):	64 copies
Amp. Efficiency (PCR Product):	94.8%
R^2 (PCR Product):	1.000

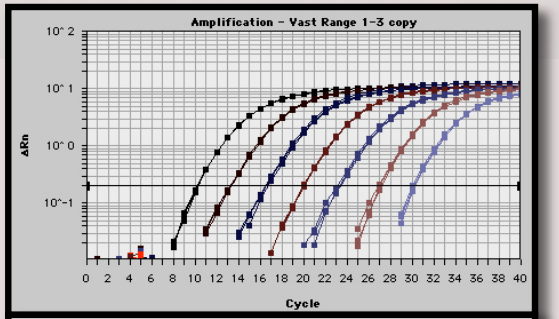
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Human Genomic DNA

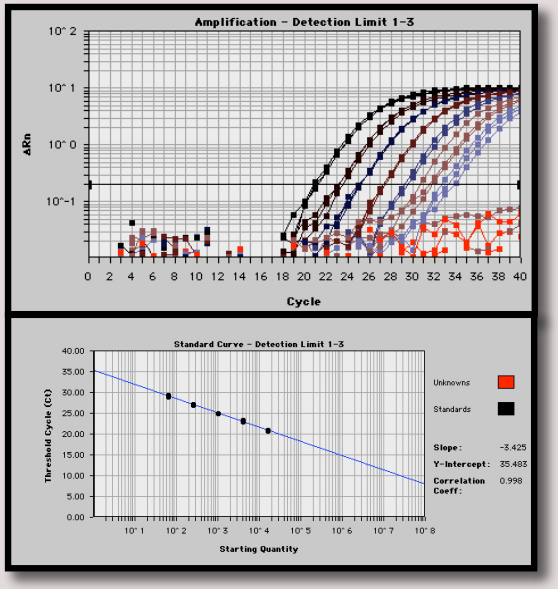


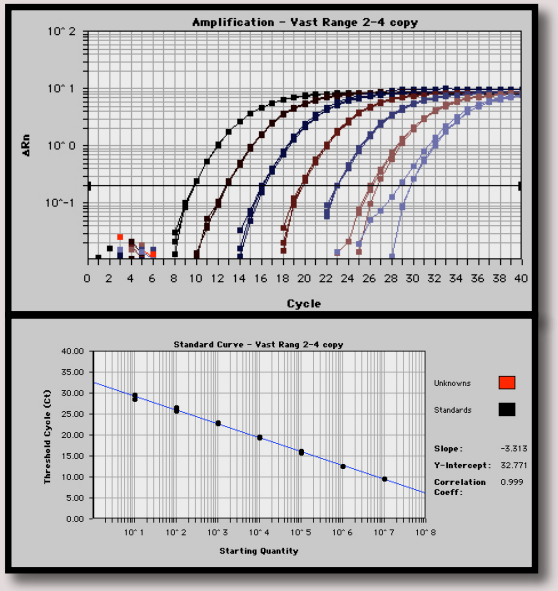
Purified PCR Product



E74-like factor 5 (ELF5)

Amp. Efficiency (genomic DNA):	101.9%
R^2 (genomic DNA):	0.998
Assay sensitivity (genomic DNA):	64 copies
Amp. Efficiency (PCR Product):	98.4%
R^2 (PCR Product):	0.999





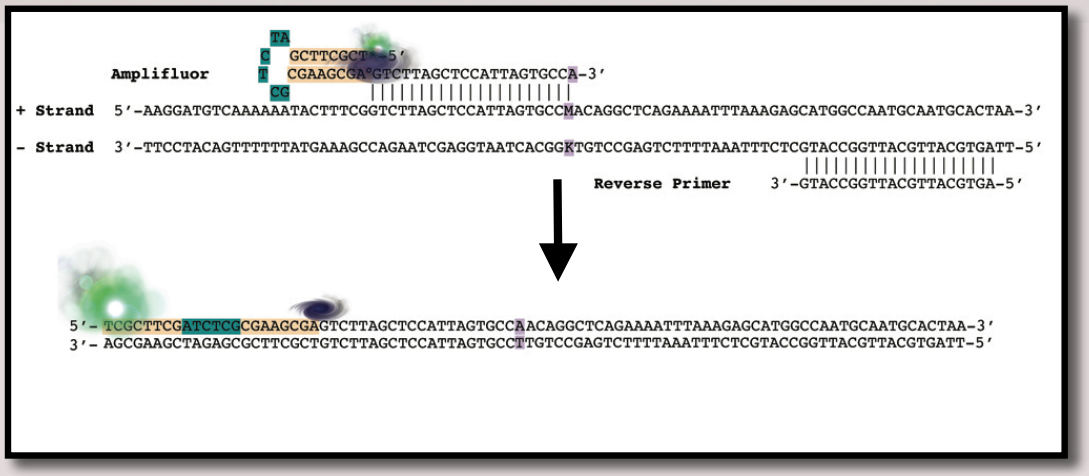
Glutathione reductase (GSR)

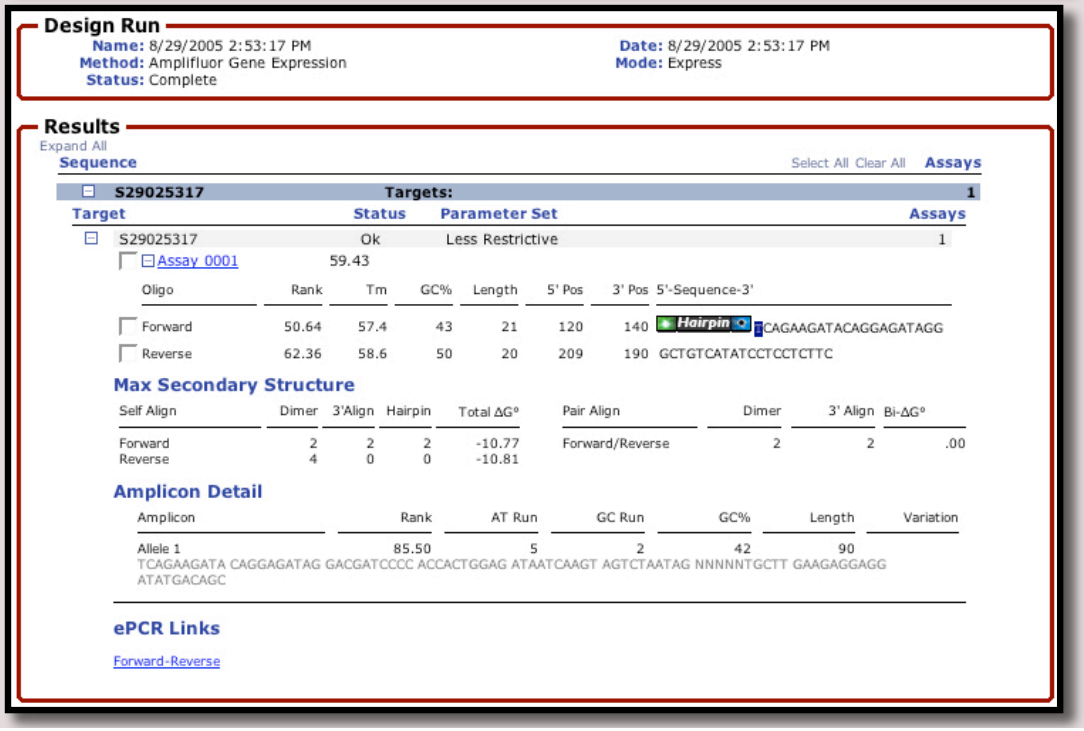
Amp. Efficiency (genomic DNA):	95.9%
R^2 (genomic DNA):	0.998
Assay sensitivity (genomic DNA):	64 copies
Amp. Efficiency (PCR Product):	100.4%
R^2 (PCR Product):	0.999

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What's Next? *Amplifluor Direct® Assay Design*





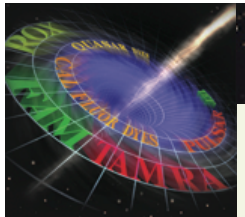
Mechanism of Action:

1) Hairpin sequence is appended to 5' end of one primer
2) Stem structure positions reporter and quencher in close proximity so that fluorscence is suppressed
3) Upon incorporating primer into amplicon, polymerase extends through hairpin sequence
4) Replicated strand spreads apart reporter and quencher, releasing fluorescence

Current Applications:

1) Interrogation of SNPs
2) *In Vitro* Diagnostic assays for pathogen detection

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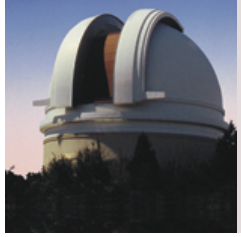
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I would like to acknowledge Raymond Peterson, Dean Fiala, and the rest of the team at Celadon Laboratories for their sophisticated expertise in developing this program, and especially their patience accomodating our many requests.

Greg Shipley has provided critical insight and suggestions regarding the features of this program, and toward refining its ease-of-use. For this I am grateful.

Finally, I would like to acknowledge *A-Z of Quantitative PCR* by Stephen Bustin. The information contained within was essential during the fine-tuning of parameter values.



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