Development of a Real Time PCR-based Bioassay for Human Interferon β

Larisa O. Miller

Third Annual CELL BASED ASSAYS

October 3rd 2007 – Berkeley, CA

biogen idec

Presentation Outline

- Interferon β (IFNβ) – Pharmacology
- IFNβ Specific Activity - Measurement
- RT-PCR Assay - Development Approach
- Results
- Data comparison
- Conclusions

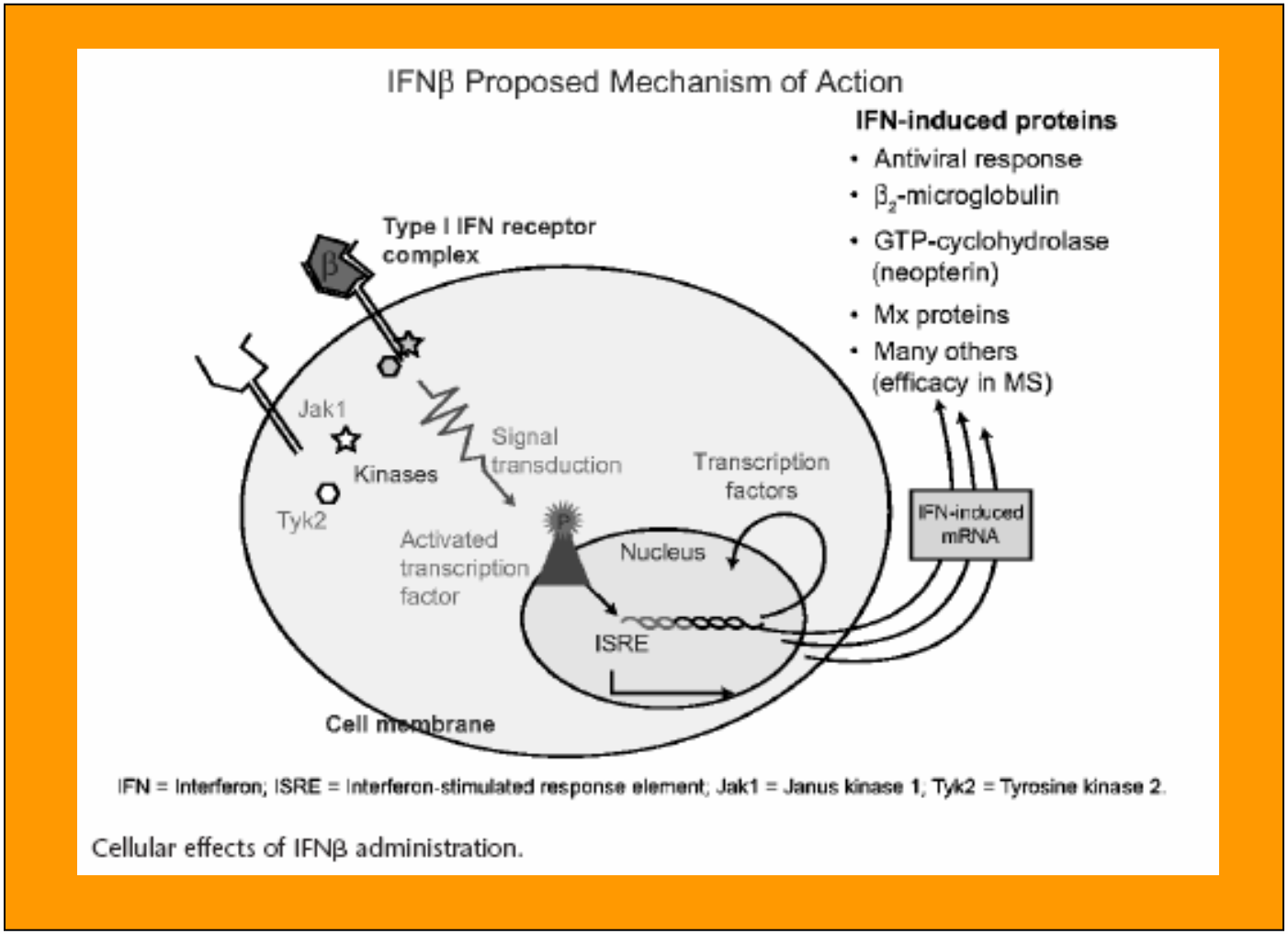
Pharmacology of IFNβ

- Interferons are a family of naturally occurring proteins and glycoproteins that are produced by eukaryotic cells in response to viral infection and other biological inducers.
- IFNβ is produced by various cell types including fibroblasts and macrophages.
- Interferons mediate antiviral, antiproliferative and immunomodulatory activities in response to viral infection and other biological inducers.

Pharmacology of IFNβ

- IFNβ exerts its biological effects by binding to specific receptors on the surface of human cells (IFNAR).
- The specific interferon-induced proteins and mechanisms by which IFNβ exerts its effects in multiple sclerosis have not been fully defined.
- The binding of IFNβ initiates a complex cascade of intracellular events that leads to the expression of numerous interferon-induced gene products and markers, including 2', 5'-oligoadenylate synthetase (OAS), β2-microglobulin, Myxovirus resistance proteins (MxA) and neopterin.
- These products have been measured in the serum and cellular fractions of blood collected from patients treated with IFNβ-1a (AVONEX®).

Pharmacology of IFNb



Pachner, AR, Multiple Sclerosis 2007; 13: S49-S52

biogen idec

IFNβ-1a Specific Activity

- **Standard** - World Health Organization (WHO) natural interferon beta standard, Second International Standard for Interferon, Human Fibroblast (Gb-23-902-531),
- **Assay -** *in vitro* CytoPathic Effect bioassay (CPE) using lung carcinoma cells (A549) and EncephaloMyoCarditis virus (EMC).
- IFNβ–1a (AVONEX®) contains approximately 200 million IU of antiviral activity per mg, using this method.

Clinical application of the CPE Assay

- Bioavailability of IFNβ in human serum (PK, NAbs)
- Measured by ELISA or CPE
- Sensitivity - CPE assay vs. ELISA
- Levels of IFNβ–1a after dose are often too low to be measured in standard ELISA

CPE Assay

Based on the ability of IFNβ to protect human lung carcinoma cells (A549) from cytopathic effect (CPE) caused by the Encephalomyocarditis (EMC) virus.

Day 1:

A549 cells are added to the wells of an assay plate(s). Plates are incubated for 20-24 hours at 37±1 °C / 5%±1 % CO2.

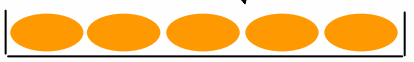
IFNβ

Day 2:

Standards, samples and controls are added to the assay plates and incubated for 15-20 hours.

EMC virus

Day3:



The EMC virus is added to all standard, samples, controls and virus control wells and incubated further for 30±1 hours.

Substrate

Day 4:

Colorimetric substrate is added to all the assay plates and incubated at 37±1 °C / 5% CO2 for additional 2 to 4 hours. Plate reader is used to record absorbance at 490 nm.

WHO Expert Committee on Biological Standardisation. Thirty-Fifth Report, 1985. WHO Technical Report Series 725. World Health Organization, Geneva.

‘Pros’ and ‘Cons’ of CPE Assay

‘Pros’

- ‘Low tech’ assay – no specialized equipment is needed (but specialized personnel and lab is)
- Relatively low cost of reagents

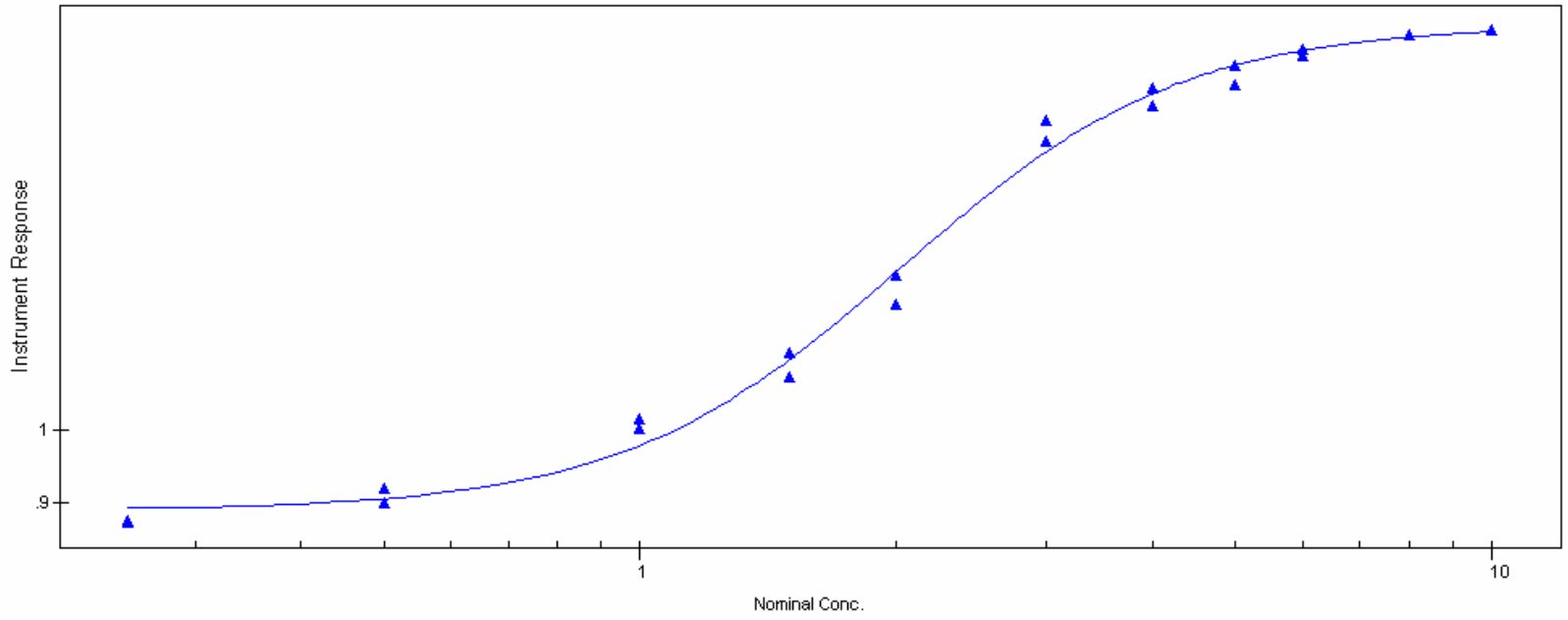
‘Cons’

- Time consuming
- Poor reproducibility
- Many variables
- Narrow range of Standard Curve
- Poor sensitivity
- Difficult outsourcing

Standard Curve Example - CPE Assay

IFNβ-1a Standard Curve in 10% Human Serum

(MTS-colorimetric read-out)
Assay range: ~1-5 IU/mL



biogen idec

MxA gene expression in response to IFNβ

Several assays based on this mechanism are described in literature

- MxA protein induction assay (MPA) - quantifying an antiviral protein (MxA for myxovirus resistant protein A) specifically induced by IFNβ

Files, J.G., Gray, J.L., Do, L.T., Foley, W.P., Gabe, J.D., Nestaas, E., Pungor Jr., E., 1998. A novel sensitive and selective bioassay for human type I interferons. J. Interferon Cytokine Res. 18, 1019.

Pungor Jr., E., Files, J.G., Gabe, J.D., Do, L.T., Foley,W.P., Gray, J.L., Nelson, J.W., Nestaas, E., Taylor, J.L., Grossberg, S.E., 1998. A novel bioassay for the determination of neutralizing antibodies to IFN-beta1b. J. Interferon Cytokine Res. 18, 1025.

- Neutralizing antibody detection in real time based PCR assay

A. Bertolotto, A. Sala, M. Caldano, M. Capobianco, S. Malucchi, F. Marnetto, F. Gilli 2007. Development and validation of a real time PCR-based bioassay for quantification of neutralizing antibodies against human interferon-beta. J. Immunol. Meth. 321, 19.

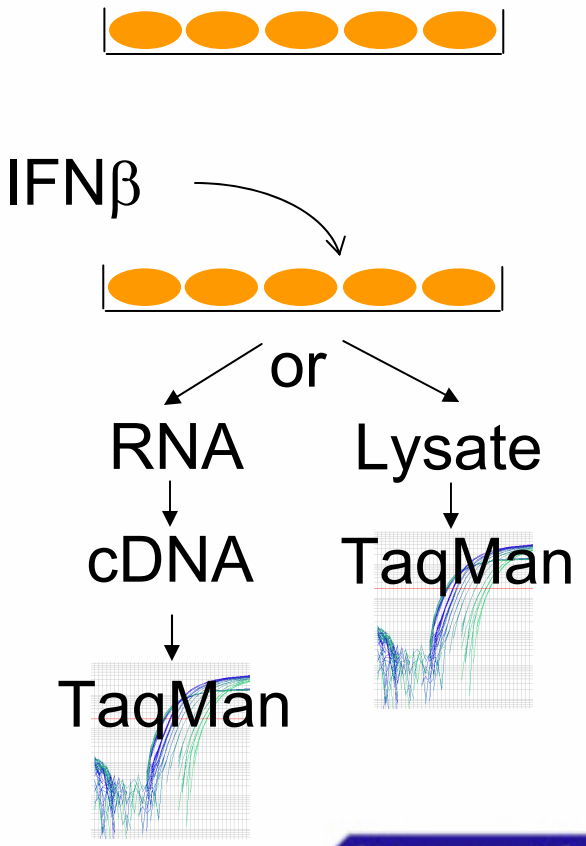
IFNβ: qRT-PCR Assay Design

Based on the ability of IFNβ to bind to the specific receptors on human lung carcinoma cells (A549) and induce cascade of intracellular events resulting in up-regulation of specific genes (MxA, OAS, etc).

Day 1: A549 cells are added to the wells of an assay plate(s). Plates are incubated overnight at 37±1 °C / 5±1% CO2.

Day 2: IFNβ is added to the assay plates and incubated for 4-5 hours. Cells are lysed and TaqMan reactions are performed directly on the cell lysates or, alternatively extracted RNA is Reverse-Transcribed overnight and,

Day3: TaqMan reactions are performed on cDNA.



biogen idec

‘Pros’ and ‘Cons’ of qRT-PCR Assay

‘Pros’

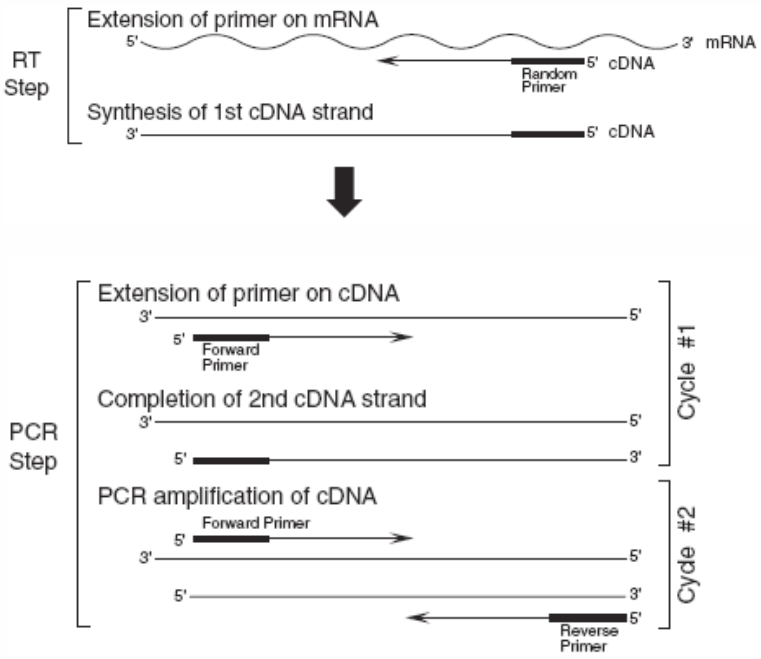
- Capturing ‘early event’ (mRNA levels) = reduced variability
- Normalizing with internal Endogenous control (GAPDH) = control for well to well variability due to different cell densities, RNA isolation and reverse transcription efficiencies and RNA degradation
- Pre-optimized reagents readily available
- Reduced processing time and increased throughput
- Increased Standard curve range and sensitivity

‘Cons’

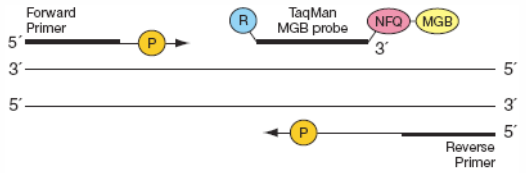
- Specialized equipment is needed to perform the assay
- Cost of initial setup (equipment and training)
- Cost of reagents

Principle of qRT-PCR

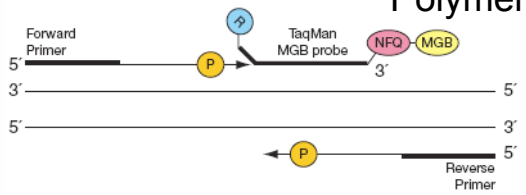
Two-step RT-PCR



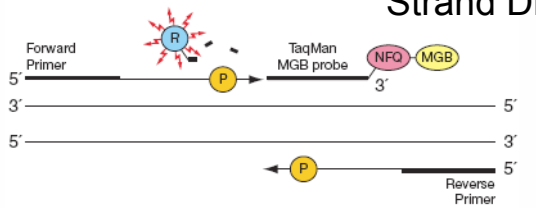
The 5'-Nuclease Assay - TaqMan



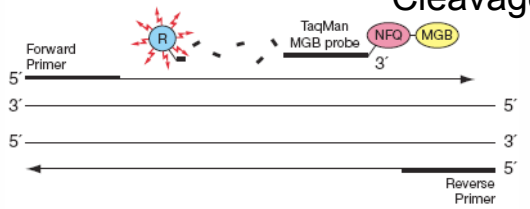
Polymerization



Strand Displacement



Cleavage



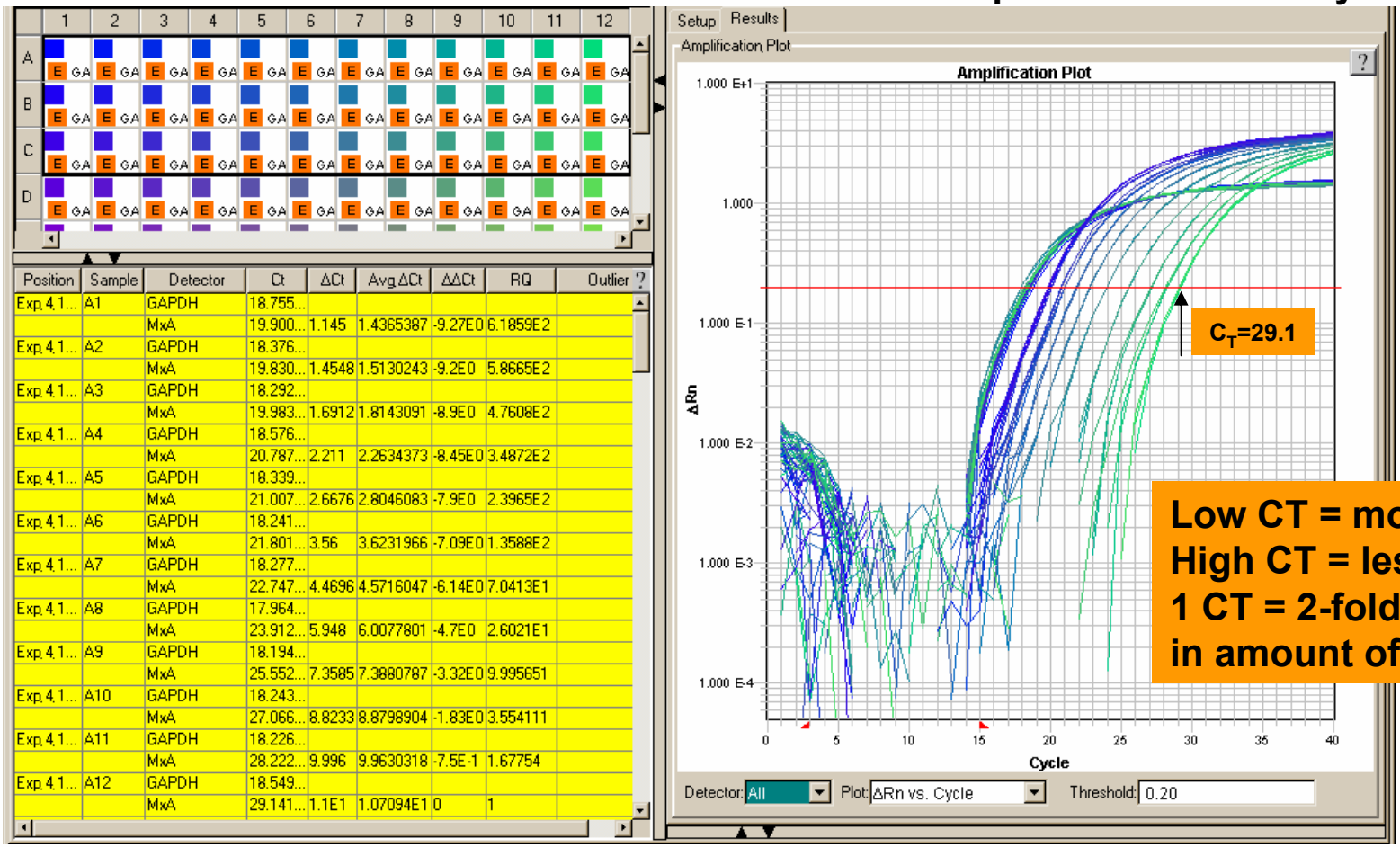
Completion of Polymerization

NFQ = Nonfluorescent quencher
MGB = Minor groove binder
R = Reporter
P = AmpliTaq Gold DNA Polymerase, UP

biogen idec

Analysis of Real-Time PCR Data

ABI Prism 7900HT
Sequence Detection System (SDS)



Position	Sample	Detector	Ct	ΔCt	Avg ΔCt	ΔΔCt	RQ	Outlier
Exp 4 1...	A1	GAPDH	18.755...					
		MxA	19.900...	1.145	1.4365387	-9.27E0	6.1859E2	
Exp 4 1...	A2	GAPDH	18.376...					
		MxA	19.830...	1.4548	1.5130243	-9.2E0	5.8665E2	
Exp 4 1...	A3	GAPDH	18.292...					
		MxA	19.983...	1.6912	1.8143091	-8.9E0	4.7608E2	
Exp 4 1...	A4	GAPDH	18.576...					
		MxA	20.787...	2.211	2.2634373	-8.45E0	3.4872E2	
Exp 4 1...	A5	GAPDH	18.339...					
		MxA	21.007...	2.6676	2.8046083	-7.9E0	2.3965E2	
Exp 4 1...	A6	GAPDH	18.241...					
		MxA	21.801...	3.56	3.6231966	-7.09E0	1.3588E2	
Exp 4 1...	A7	GAPDH	18.277...					
		MxA	22.747...	4.4696	4.5716047	-6.14E0	7.0413E1	
Exp 4 1...	A8	GAPDH	17.964...					
		MxA	23.912...	5.948	6.0077801	-4.7E0	2.6021E1	
Exp 4 1...	A9	GAPDH	18.194...					
		MxA	25.552...	7.3585	7.3880787	-3.32E0	9.995651	
Exp 4 1...	A10	GAPDH	18.243...					
		MxA	27.066...	8.8233	8.8798904	-1.83E0	3.554111	
Exp 4 1...	A11	GAPDH	18.226...					
		MxA	28.222...	9.996	9.9630318	-7.5E-1	1.67754	
Exp 4 1...	A12	GAPDH	18.549...					
		MxA	29.141...	1.1E1	1.07094E1	0	1	

Low CT = more RNA
High CT = less RNA
1 CT = 2-fold difference in amount of template

Experimental Data

biogen idec

Absolute vs. Relative Quantitation

- Absolute quantitation requires that the absolute quantities of the standard be known by some independent means.
- Plasmid DNA or *in vitro* transcribed RNA are commonly used to prepare absolute standards. Concentration is measured by A260 and converted to the number of copies using the molecular weight of the DNA or RNA.

- Relative quantitation - quantity is expressed relative to some basis sample, such as the calibrator. For all experimental samples, target quantity is determined from the standard curve and divided by the target quantity of the calibrator. Thus, the calibrator becomes the 1 ×sample, and all other quantities are expressed as an *n*-fold difference relative to the calibrator. For example, in a study of drug effects on gene expression, the untreated control would be an appropriate calibrator.

- $\Delta\Delta C_T$ method - Relative quantitation can be performed using this method instead of relative standard curve. It is necessary to demonstrate that efficiencies of target and reference amplification are approximately equal.

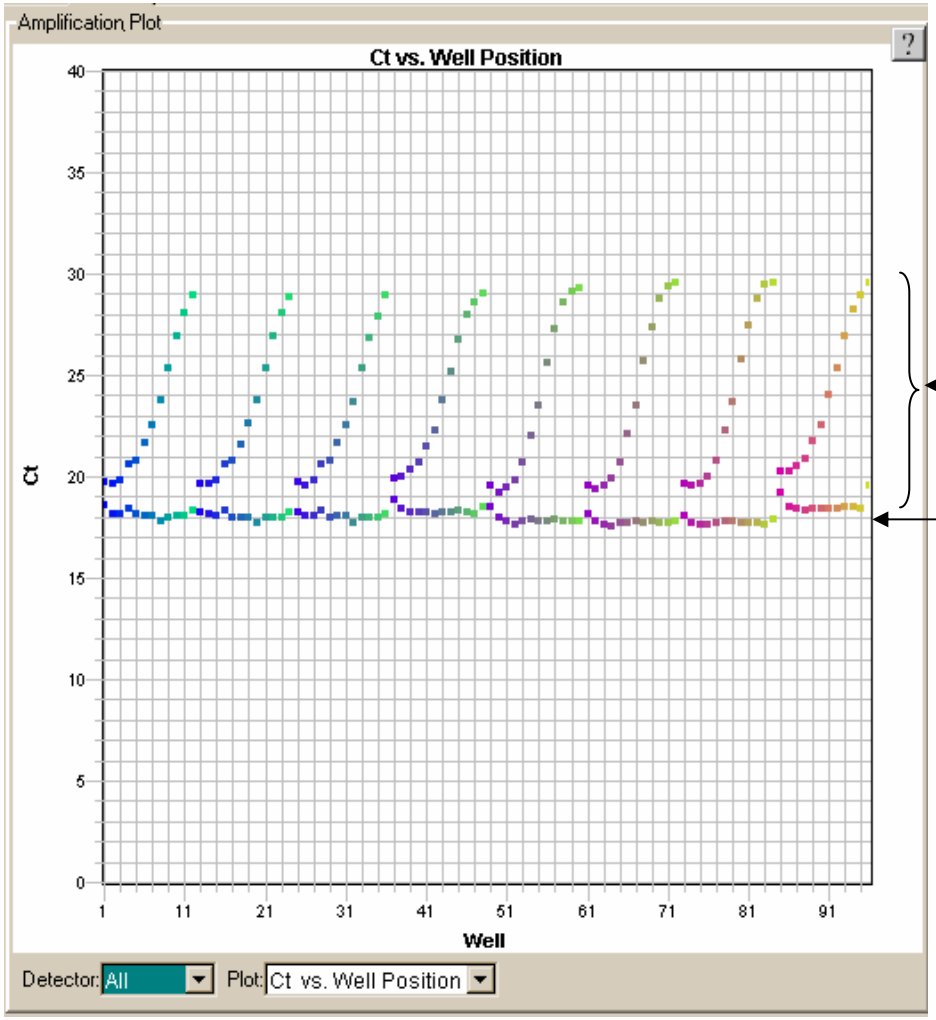
Principle of Relative Quantitation

•Amplification of an endogenous control is performed to standardize the amount of sample RNA or DNA added to a reaction. Examples of such genes are ß-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal RNA (18S).

•**Endogenous control** – an RNA or DNA that is present in each experimental sample as isolated. By using an endogenous control as an active reference, you can normalize quantitation of a messenger RNA (mRNA) target for differences in the amount of total RNA added to each reaction.

•**Calibrator** - A sample used as the basis for comparative results.

Normalization with Endogenous Control (GAPDH)



Bi-plex reactions:
Determination of MxA levels normalized with GAPDH

MxA response

GAPDH levels

Experimental Data

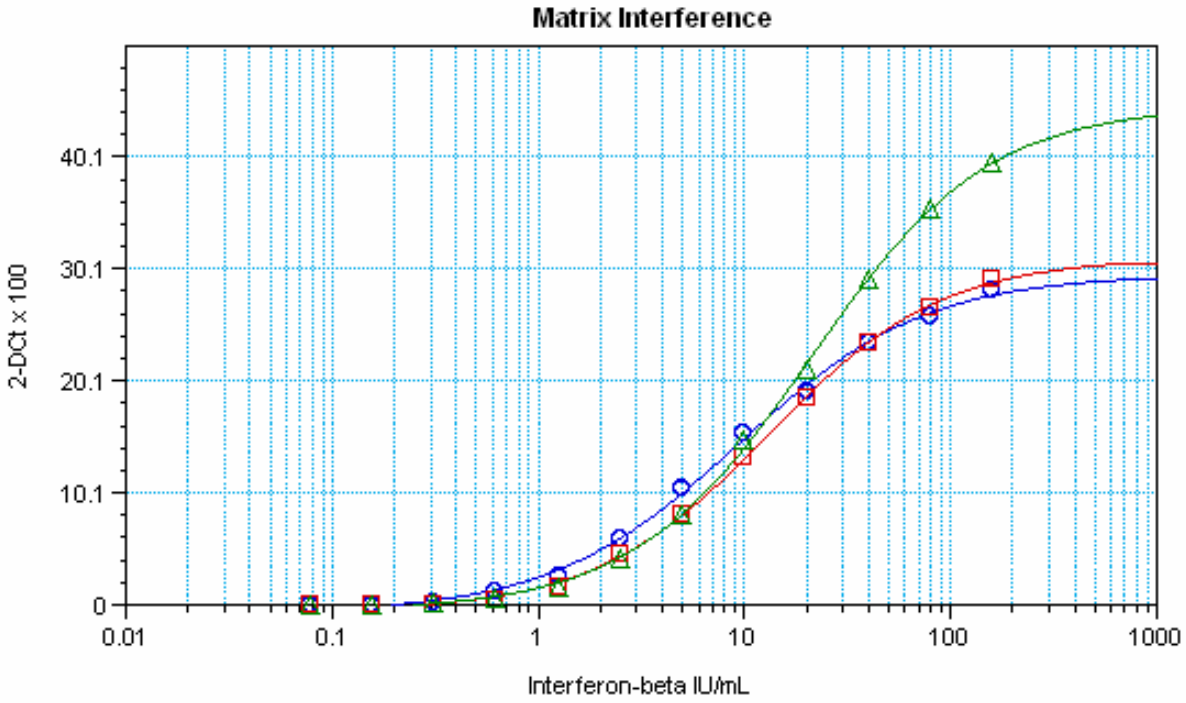
biogen idec

CPE qRT-PCR Assay Development

- Targets:
  - IFNβ–1a
  - Modified IFNβ–1a
- Matrix effect
  - Normal Human Serum (NHS)
- Time-course
- OAS2 vs. MxA expression
- Two step vs. One step read-out
- Assay performance - Clinical sample testing

Matrix Effect : IFNβ–1a

NHS effect (2-10%) on IFNβ-induced MxA expression



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
○ Plot#1 (IFN-beta 1a 2 %: Concentration vs Values)	-0.626	0.958	9.39	29.6	0.999
□ Plot#2 (IFN-beta 1a 5%: Concentration vs Values)	-0.479	1.03	13.1	31.1	0.999
△ Plot#3 (IFN-beta 1a 10%: Concentration vs Values)	-0.416	1.01	20.9	44.6	1

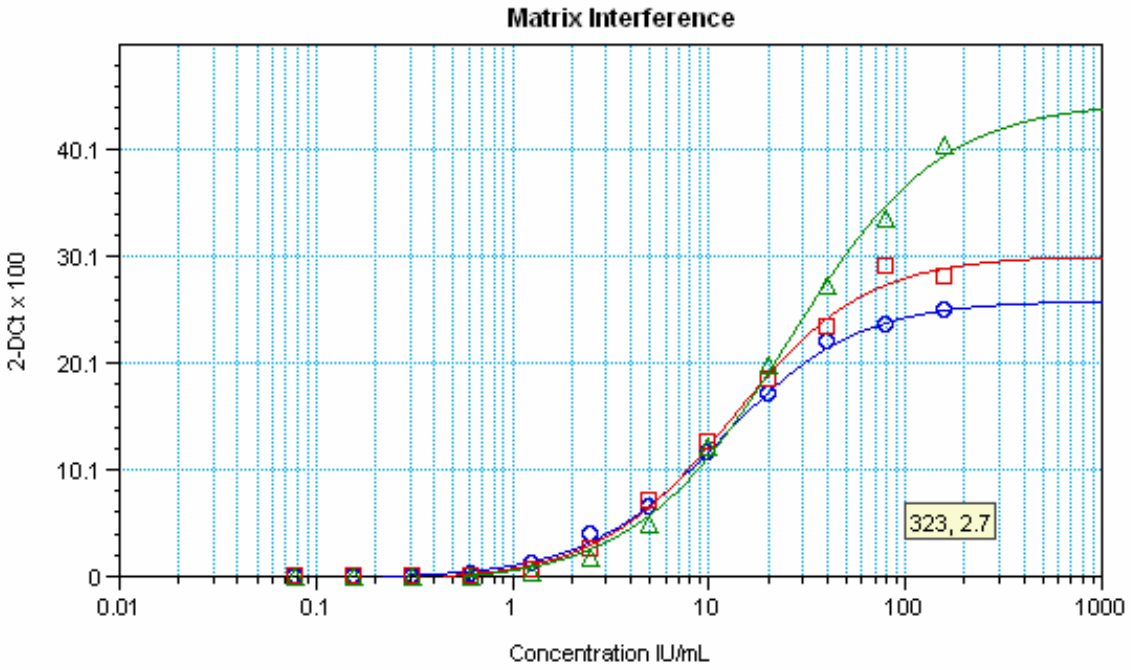
Better dynamic range (10%)

Superimposed curves (2% and 5%)

biogen idec

Matrix Effect: Modified IFNβ-1a

NHS effect (2-10%) on IFNβ-induced MxA expression



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
○ Plot#1 (Modified IFN-beta 1a 2%: Concentration vs ...	-0.0844	1.25	11.5	26	0.999
□ Plot#2 (Modified IFN-beta 1a 5%: Concentration vs ...	-0.311	1.27	13.1	30.2	0.996
△ Plot#3 (Modified IFN-beta 1a 10%: Concentration v...	-0.557	1.12	25.1	44.7	0.998

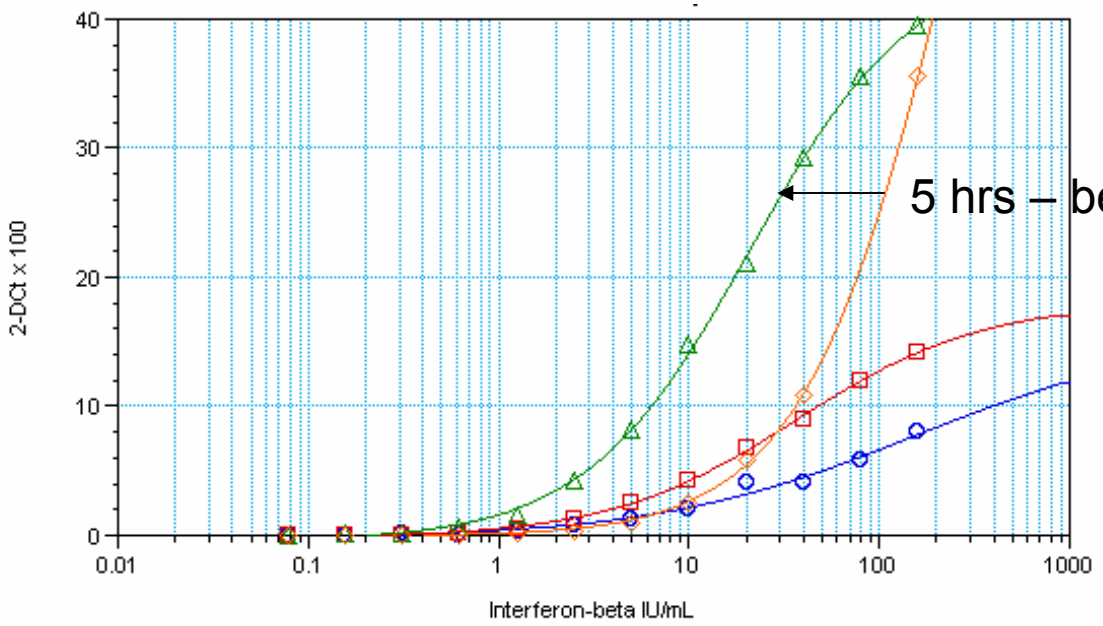
Better dynamic range (10%)

Comparable curves (2% and 5%)

biogen idec

Time-course of IFNβ-1a induced MxA expression

MxA expression after 2, 3, 5 and 24 hrs of exposure



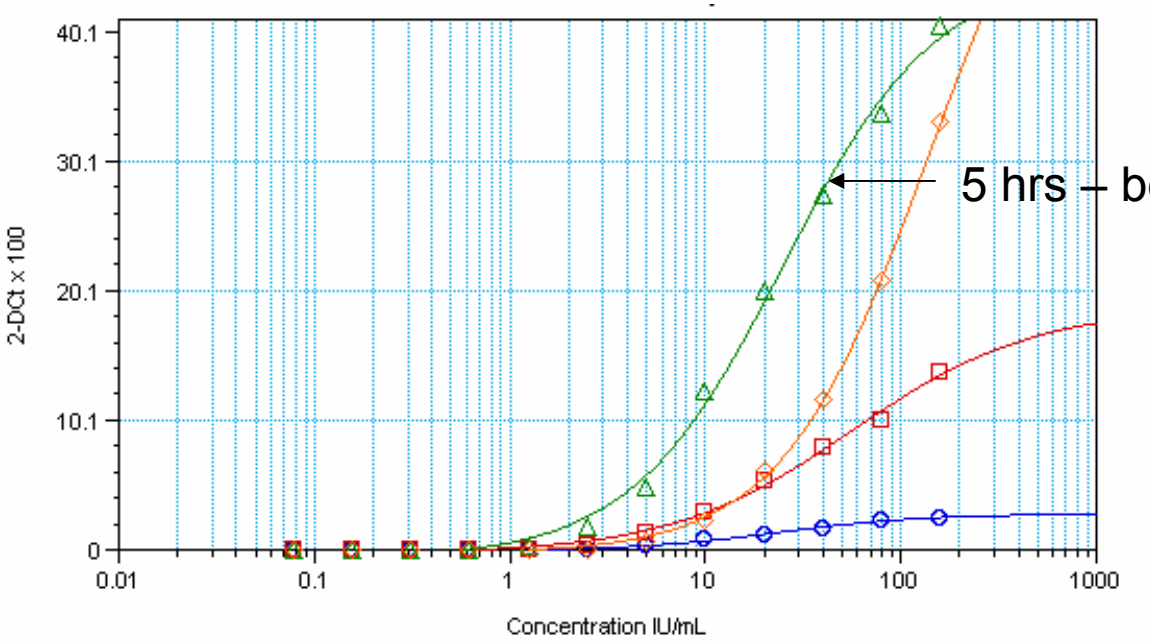
5 hrs – best sensitivity and dynamic range

4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
Plot#1 (IFN-beta 1a 2 hrs: Concentration vs Values)	-0.244	0.633	155	15.7	0.986
Plot#2 (IFN-beta 1a 3 hrs: Concentration vs Values)	-0.198	0.883	37	18.1	0.999
Plot#3 (IFN-beta 1a 5 hrs: Concentration vs Values)	-0.416	1.01	20.9	44.6	1
Plot#4 (IFN-beta 1a 24 hrs: Concentration vs Values)	-0.0869	1.08	267	97.9	1

biogen idec

Time-course of Modified IFNβ–1a induced MxA expression

MxA expression after 2, 3, 5 and 24 hrs of exposure



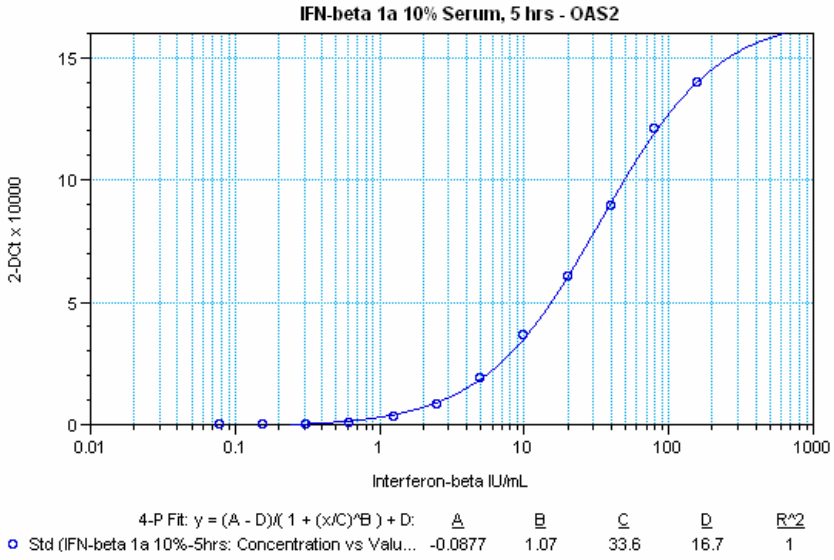
5 hrs – best sensitivity and dynamic range

4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
○ Plot#1 (Modified IFN-beta 1a 2 hrs: Concentration v...	-0.0141	1.13	23.9	2.91	0.995
□ Plot#2 (Modified IFN-beta 1a 3 hrs: Concentration v...	-0.204	0.93	56.8	18.8	0.996
△ Plot#3 (Modified IFN-beta 1a 5 hrs: Concentration v...	-0.557	1.12	25.1	44.7	0.998
◇ Plot#4 (Modified IFN-beta 1a 24 hrs: Concentration ...	-0.16	1.16	138	60.9	0.999

biogen idec

IFNβ–1a induced MxA and OAS2 expression

OAS2 expression

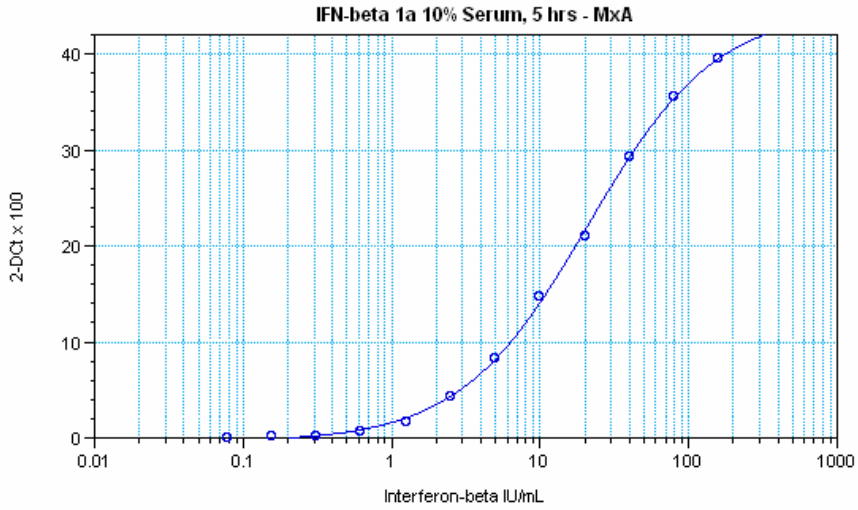


4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
Std (IFN-beta 1a 10%-5hrs: Concentration vs Valu...	-0.0877	1.07	33.6	16.7	1

IFN-beta 1a 10%-5hrs (IU/ml)

Sample	Concentration	Values	Backfit	%Return
St01	160.000	13.983	111.516	106.315
St02	80.000	12.086	61.017	94.291
St03	40.000	8.962	33.630	101.470
St04	20.000	6.016	15.847	101.241
St05	10.000	3.628	7.316	103.122
St06	5.000	1.918	2.810	91.100
St07	2.500	0.831	1.016	76.288
St08	1.250	0.308	0.471	77.493
St09	0.625	0.084	0.335	113.197
St10	0.313	0.027	0.304	206.395
St11	0.156	0.019	0.285	388.982
St12	0.078	0.010	0.272	743.474

MxA expression



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
Std (IFN-beta 1a 10%-5hrs: Concentration vs Valu...	-0.416	1.01	20.9	44.6	1

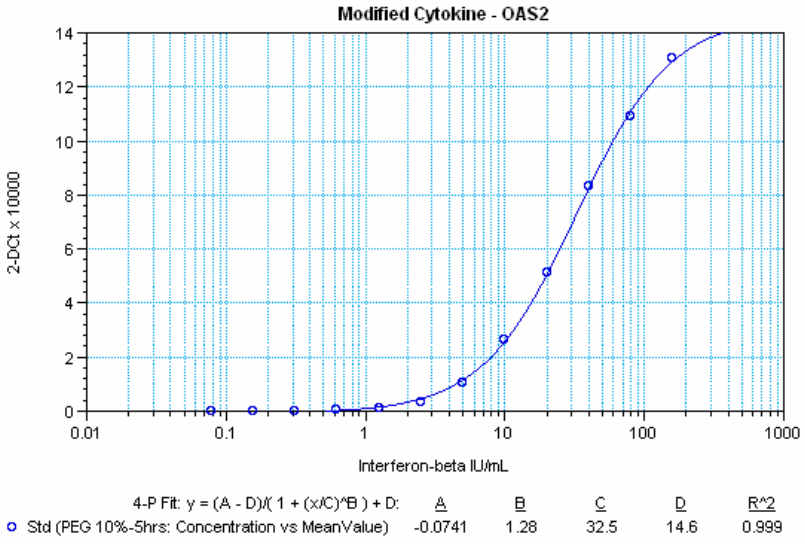
IFN-beta 1a 10%-5hrs (IU/ml)

Sample	Concentration	Backfit	Values	%Return
St01	160.000	159.747	39.520	99.842
St02	80.000	81.137	35.512	101.422
St03	40.000	40.015	29.254	100.038
St04	20.000	19.064	21.081	95.321
St05	10.000	10.682	14.773	106.817
St06	5.000	5.026	8.235	100.514
St07	2.500	2.492	4.306	99.674
St08	1.250	0.993	1.576	79.429
St09	0.625	0.499	0.601	79.811
St10	0.313	0.308	0.215	98.614
St11	0.156	0.251	0.099	160.855
St12	0.078	0.233	0.061	297.931

biogen idec

Modified IFNβ–1a induced MxA and OAS2 expression

OAS2 expression

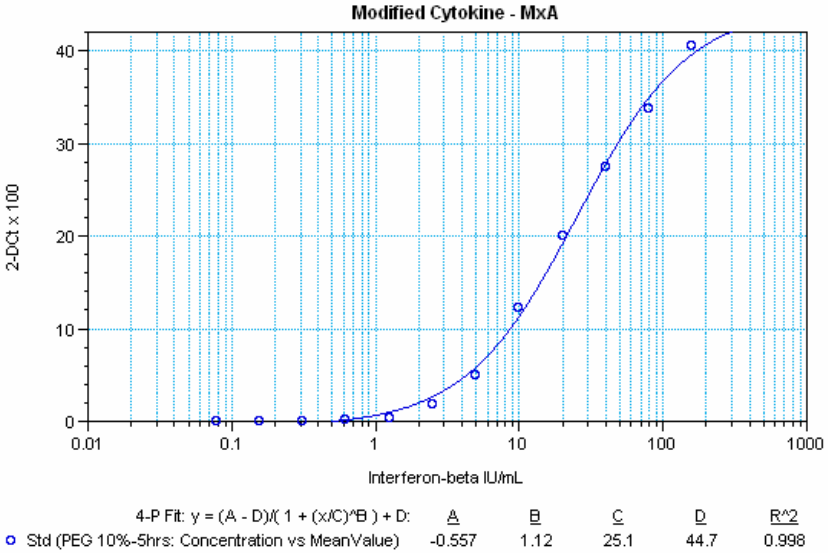


4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
Std (PEG 10%-5hrs: Concentration vs MeanValue)	-0.0741	1.28	32.5	14.6	0.999

Modified IFN-beta 1a 10%-5hrs (IU/ml)

Sample	Concentration	Values	Backfit	%Return
St01	160.000	13.054	170.104	106.315
St02	80.000	10.898	75.433	94.291
St03	40.000	8.316	40.588	101.470
St04	20.000	5.113	20.248	101.241
St05	10.000	2.671	10.312	103.122
St06	5.000	1.022	4.555	91.100
St07	2.500	0.304	1.907	76.288
St08	1.250	0.087	0.969	77.493
St09	0.625	0.034	0.707	113.197
St10	0.313	0.022	0.645	206.395
St11	0.156	0.015	0.608	388.982
St12	0.078	0.010	0.581	743.474

MxA expression



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
Std (PEG 10%-5hrs: Concentration vs MeanValue)	-0.557	1.12	25.1	44.7	0.998

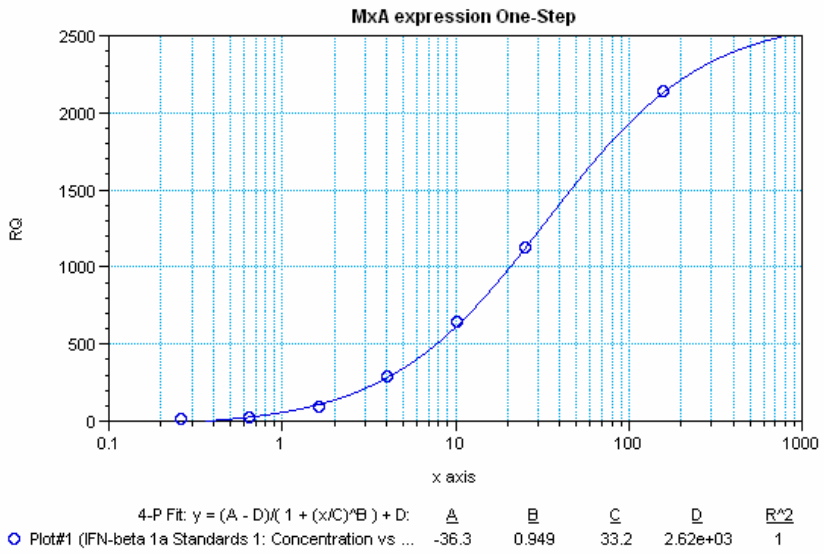
Modified IFN-beta 1a 10%-5hrs (IU/ml)

Sample	Concentration	Values	Backfit	%Return
St01	160.000	40.495	193.577	120.986
St02	80.000	33.765	69.943	87.428
St03	40.000	27.440	38.720	96.801
St04	20.000	19.994	21.294	106.469
St05	10.000	12.305	10.983	109.835
St06	5.000	4.984	4.308	86.152
St07	2.500	1.848	1.907	76.277
St08	1.250	0.401	0.812	64.989
St09	0.625	0.135	0.604	96.644
St10	0.313	0.050	0.536	171.613
St11	0.156	0.032	0.522	333.987
St12	0.078	0.031	0.521	666.946

biogen idec

One Step vs. Two Step read-out: IFNβ-1a

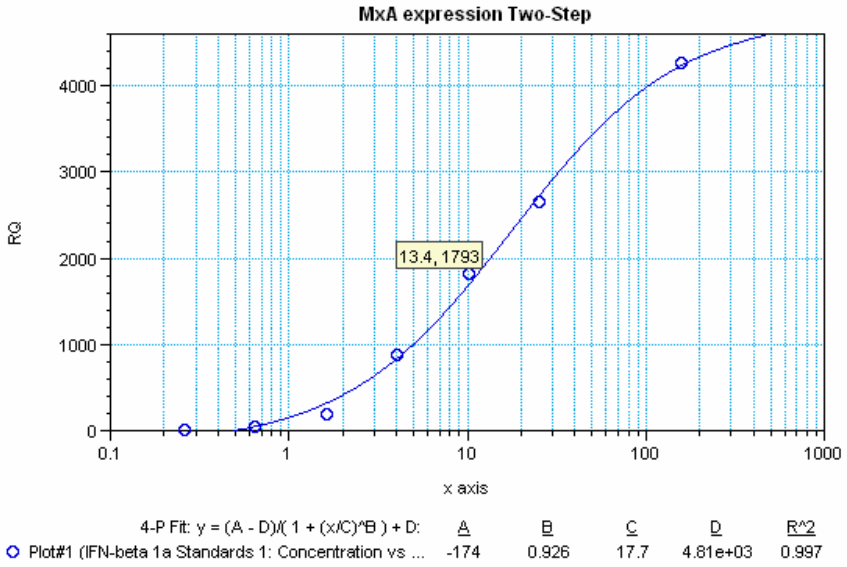
‘One-Step’ process:
Cell Lysis > One Step RT-PCR



IFN-beta 1a Standards 1 (IU/ml)

Sample	Concentration	Values	Backfit	%Return
Av01	160.000	2135.179	160.626	100.39
Av02	64.000	Masked	Masked	Masked
Av03	25.600	1119.014	25.159	98.28
Av04	10.240	638.468	10.657	104.07
Av05	4.096	285.393	4.109	100.31
Av06	1.638	84.077	1.337	81.60
Av07	0.655	21.828	0.605	92.37
Av08	0.262	8.529	0.468	174.70

‘Two-Step’ process:
RNA isolation > RT > TaqMan



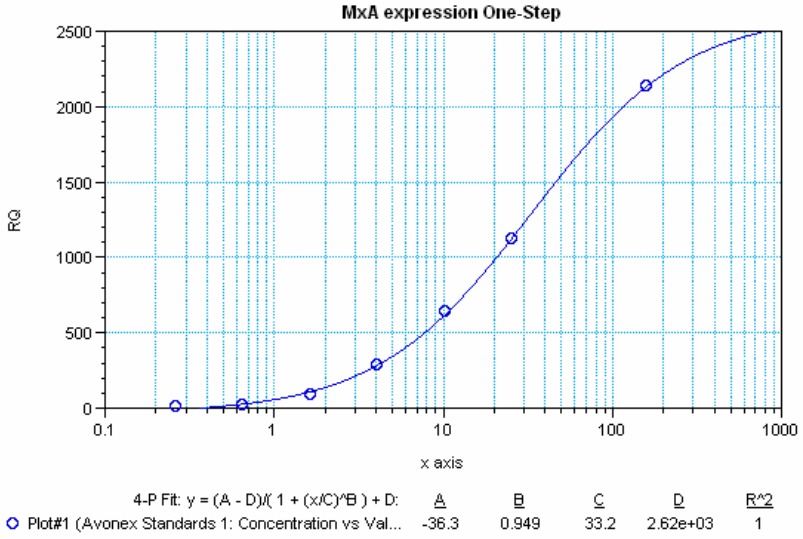
IFN-beta 1a Standards 1 (IU/ml)

Sample	Concentration	Values	Backfit	%Return
Av01	160.000	4254.900	166.721	104.20
Av02	64.000	Masked	Masked	Masked
Av03	25.600	2645.220	23.588	92.14
Av04	10.240	1817.673	11.429	111.61
Av05	4.096	869.034	4.224	103.12
Av06	1.638	179.388	1.104	67.37
Av07	0.655	36.819	0.612	93.38
Av08	0.262	7.810	0.518	197.72

biogen idec

One Step vs. Two Step read-out: Modified IFNβ-1a

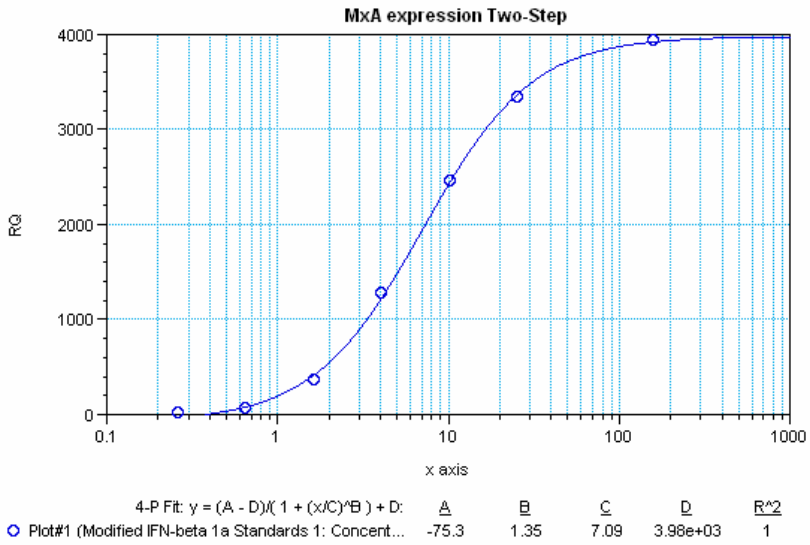
‘One-Step’ process:
Cell Lysis > One Step RT-PCR



Modified IFN-beta 1a Standards 1 (IU/mL)

Sample	Concentration	Values	Backfit	%Return
PE01	160.000	2649.8	153.601	96.00
PE02	64.000	Masked	Masked	Masked
PE03	25.600	2270.0	26.214	102.40
PE04	10.240	1604.3	9.983	97.49
PE05	4.096	853.37	4.240	103.53
PE06	1.638	275.06	1.565	95.50
PE07	0.655	55.039	0.584	89.06
PE08	0.262	13.094	0.365	139.18

‘Two-Step’ process:
RNA isolation > RT > TaqMan



Modified IFN-beta 1a Standards 1 (IU/mL)

Sample	Concentration	Values	Backfit	%Return
PE01	160.000	3941.682	211.029	131.89
PE02	64.000	Masked	Masked	Masked
PE03	25.600	3342.558	24.457	95.53
PE04	10.240	2455.628	10.299	100.58
PE05	4.096	1269.898	4.221	103.06
PE06	1.638	362.511	1.488	90.80
PE07	0.655	65.230	0.606	92.44
PE08	0.262	10.356	0.416	158.62

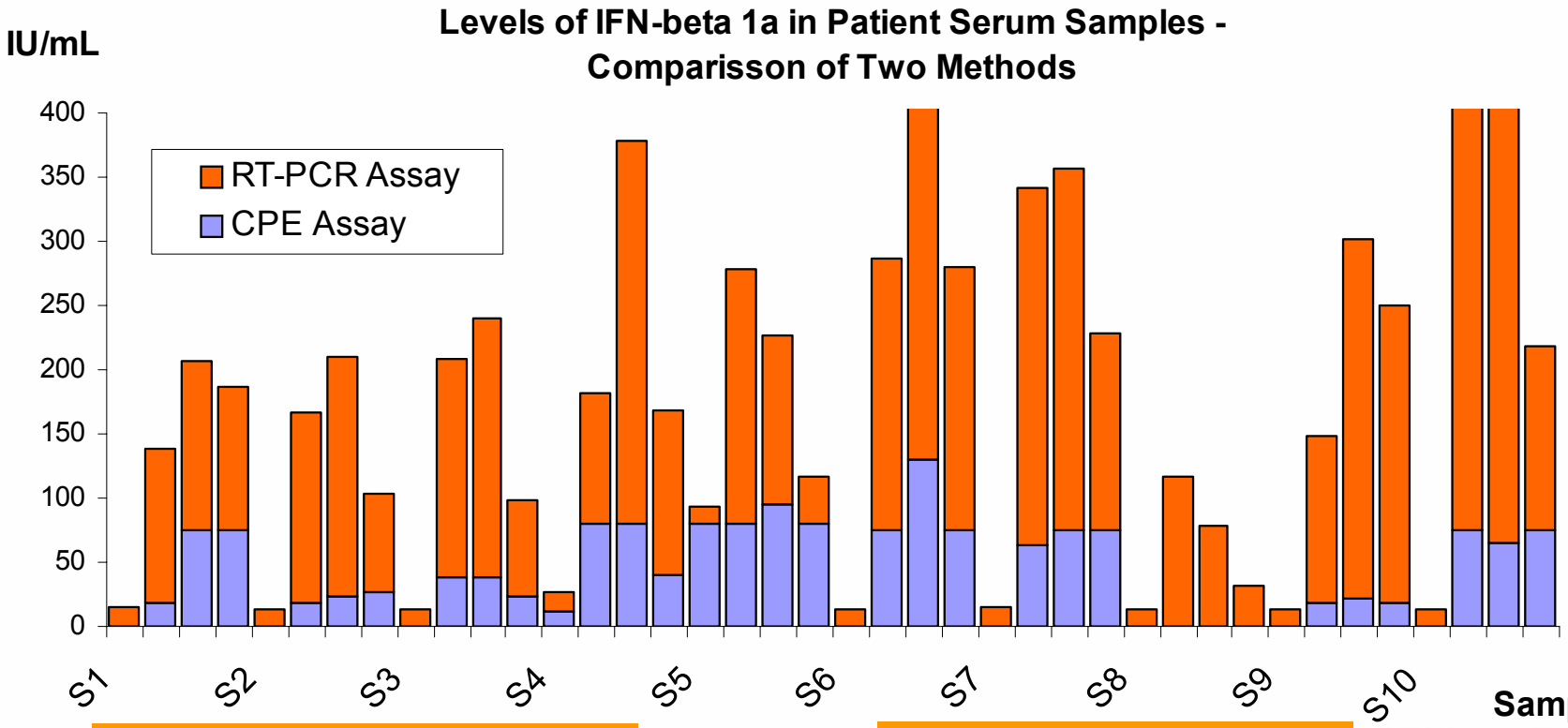
biogen idec

Optimized Assay Design

- Optimum IFNb exposure time – 4-5 hrs
- Optimum NHS concentration – 10%
- Assay performs equally well for IFNβ-1a and modified IFNβ-1a (sensitivity, dynamic range)
- OAS2 read-out has similar sensitivity and range as MxA, but MxA is chosen (literature references)
- Read-out can be performed either as One-step (lysis - TaqMan) or Two-step (RNA - cDNA - TaqMan)

Clinical Samples

- 10 patients / 4 time points each
  - pre-, 6 hrs, 12 hrs, 24 hrs
- historical data available (CPE assay)



Comparable PK profiles

Improved Sensitivity

biogen idec

Conclusions

- The IFNβ PCR based assay is
  - sensitive
  - simple
  - time efficient
  - has significant advantages over the established CPE assay.

The assay is easily transferable to a commercial testing laboratory and could be used for product release and in a clinical setting.

Acknowledgments

- Gulraiz Khan
- Michaela Lerner
- Paula Hochman
- Meena Subramanyam
- CST-NSBU

Q&A