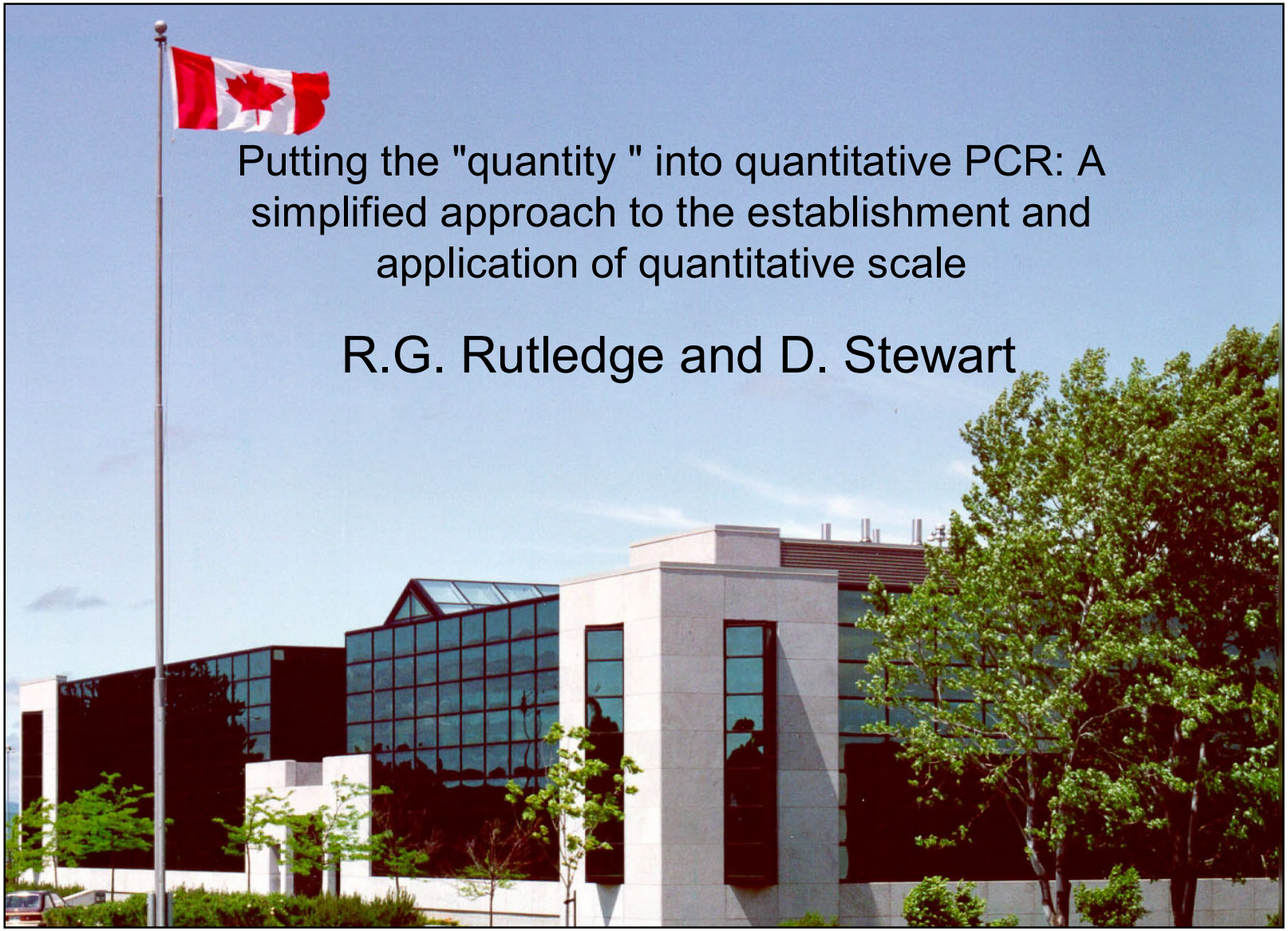

Putting the "quantity " into quantitative PCR: A simplified approach to the establishment and application of quantitative scale

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Natural Resources Canada
Ressources naturelles Canada

qPCR 2005,
Freising-Weihenstephan, Germany

Canada

Major Concepts

Establishment of quantitative scale via optical calibration

-correlating reaction fluorescence to DNA mass

-three methods based upon lambda genomic DNA and SYBR Green I

Application of a single quantitative scale to multiple amplicons

-abrogates the need to prepare a quantified standard for each target

-illustration based upon cDNA quantification

Validation of absolute scale

-verifying true accuracy-of-scale

-via the level of correlation produced by different calibration methodologies

-via Limiting Dilution Assay (independent of a quantified standard)

Quantitative Scale is Not Limited to Molecules (N)

Molecules

N_t determines quantitative scale

However, N_t is dependent on amplicon size (A_S)

$$N_0 = \frac{N_t}{(E+1)^{C_t}}$$

$$N_0 = \frac{M_0 \times 9.1x10^{11}}{A_S}$$

DNA Mass

M_t is independent of amplicon size

However, M_t is dependent on fluorescence threshold (F_t)

$$M_0 = \frac{M_t}{(E+1)^{C_t}}$$

Reaction Fluorescence

F_t is a known value

If *E* is known target quantity (F_0) can be calculated

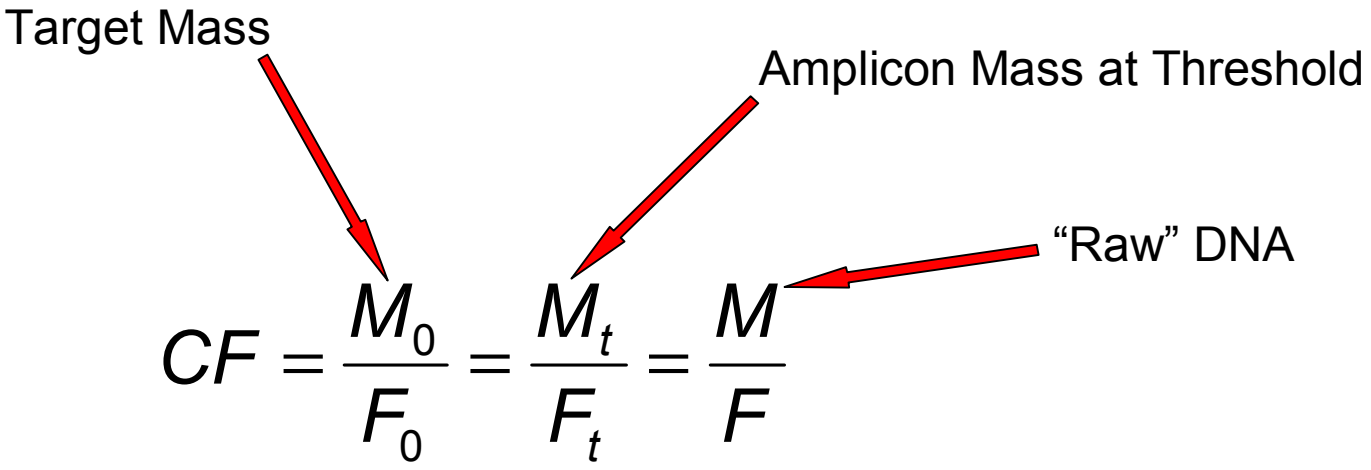
$$F_0 = \frac{F_t}{(E+1)^{C_t}}$$

$$M_0 = CF \times F_0$$

"Calibration Factor"

Optical Calibration via a Quantified Standard: Relating reaction fluorescence to DNA mass

$$CF = \frac{\text{DNA Mass}}{\text{Reaction Fluorescence}} = \frac{M}{F} = \text{ng/FU}$$

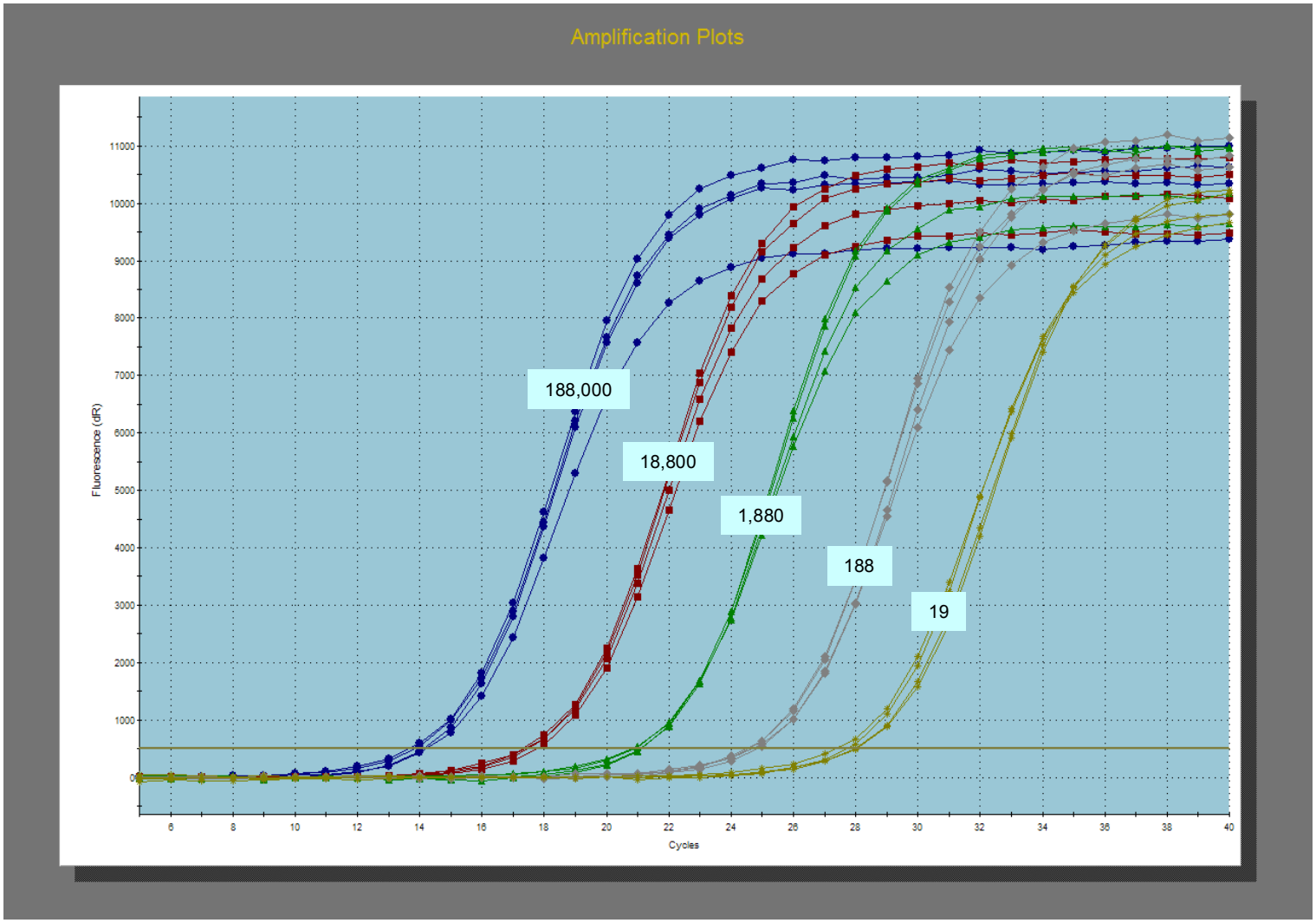


Lambda gDNA is an Effective Standard for Quantitative PCR

- Common molecular standard
- Linear
- Large genome (48,502 bp)
- Commercially available in pure form (BioLabs)

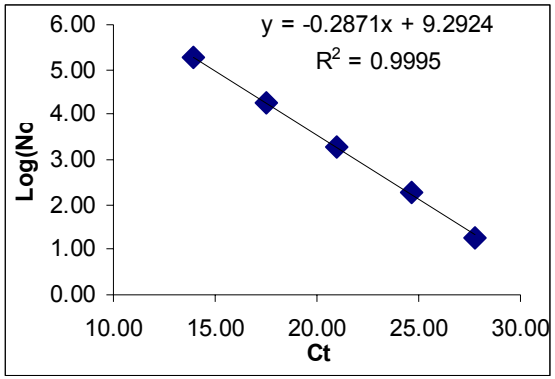
PCR-based Optical Calibration via Standard Curving

LamK7K12 10pg to 1fg Lambda gDNA



CF Determination via Standard Curving (N_t)

# of Lambda Genomes (No)	Log(No)	Av. Ct
188,000	5.274	13.95
18,800	4.274	17.48
1,880	3.274	20.99
188	2.274	24.63
19	1.274	27.78
	r2:	0.9995
	Es:	93.7%
	Nt:	**1.96E+09**
	As (bp):	150
	Mt (ng):	0.32
	Ft (FU):	500
	CF (ng/FU):	6.46E-04



$$E_S = 10^{-\text{Slope}} - 1$$

$$N_t = 10^{\text{Intercept}}$$

$$M_t = \frac{N_t \times A_S}{9.1 x 10^{11}}$$

$$CF = \frac{M_t}{F_t}$$

CF Determination via Conversion of C_t to Fo

$$F_0 = \frac{F_t}{(E+1)^{C_t}} \qquad M_0 = \frac{\text{ng Lamda DNA} \times A_S}{48{,}502} \qquad CF = \frac{M_0}{F_0}$$

# of Lambda Genomes (No)	Av. Ct	Fo (FU)
188,000	13.95	4.95E-02
18,800	17.48	4.79E-03
1,880	20.99	4.71E-04
188	24.63	4.23E-05
19	27.78	5.29E-06
	Ft:	500
	Es:	93.7%

Lambda DNA Mass (fg)	Mo (ng)
10,000	3.09E-05
1,000	3.09E-06
100	3.09E-07
10	3.09E-08
1	3.09E-09
As (bp):	150

CF (ng/FU)
6.24E-04
6.46E-04
6.57E-04
7.31E-04
5.85E-04
Av. CF: 6.49E-04
CV: 8.28%

Stanard Curve CF:	6.46E-04
Overall Av. CF:	6.47E-04

Ft (FU):	500
Mt (ng):	0.32

$$M_t = CF \times F_t$$

Relative Quantitative Accuracy: An example

$$F_0 = \frac{F_t}{(E+1)^{C_t}} \qquad M_0 = CF \times F_0 \qquad N_0 = \frac{M_0 \times 9.1x10^{11}}{A_S}$$

Ft: 500
Es: 93.7%
CF: 6.47E-04
As (bp): 150

# of Lambda Genomes (No)	Av. Ct	Fo (FU)	Mo (ng)	No	% of Predicted
188,000	13.95	4.95E-02	3.21E-05	194,447	103.4%
18,800	17.48	4.79E-03	3.10E-06	18,784	99.9%
1,880	20.99	4.71E-04	3.05E-07	1,848	98.3%
188	24.63	4.23E-05	2.74E-08	166	88.3%
19	27.78	5.29E-06	3.42E-09	21	109.2%
				SD:	7.7%

However, precision does not necessarily reflect accuracy

True accuracy is dependent on the accuracy of both the standard and E_S estimate

Verification via an alternative method for optical calibration

Direct Calibration: PCR independent

Mock amplification reactions containing 5 ng Lambda DNA

5ng Lambda, 65 oC

	A4	B4	C4	D4	E4	F4	G4	H4
Reads	No DNA	No DNA	No DNA	No DNA	5ng Lam	5ng Lam	5ng Lam	5ng Lam
1	4467	4537	4693	4642	12544	11999	12581	12373
2	4439	4523	4698	4637	12749	12156	12635	12373
3	4421	4531	4705	4637	12886	12323	12709	12423
4	4425	4528	4692	4611	12952	12424	12769	12458
5	4431	4498	4678	4591	12978	12446	12772	12468
6	4422	4497	4685	4594	12993	12449	12755	12486
7	4405	4496	4691	4612	13012	12461	12785	12562
8	4408	4496	4697	4608	13066	12517	12808	12618
9	4423	4505	4702	4580	13162	12546	12851	12654
10	4439	4509	4690	4564	13262	12576	12932	12714
Av.:	4428	4512	4693	4608	12960	12390	12760	12513
CV:	0.40%	0.35%	0.17%	0.56%	1.57%	1.47%	0.79%	0.95%

Av. Fb: 4560 **CV:** 2.52%

Av. FU: 12656 **CV:** 2.01%

Av. FU-Fb: **8095**

M (ng): 5.00

CF (ng/FU): 6.18E-04

Ft (FU): 500

Mt (ng): 0.31

$$CF = \frac{M}{F}$$

$$M_t = CF \times F_t$$

Direct Calibration: Replicate 1 month later

5ng Lambda, 65 oC

Reads	A4 No DNA	B4 No DNA	C4 No DNA	D4 No DNA	E4 5ng Lam	F4 5ng Lam	G4 5ng Lam	H4 5ng Lam
1	4124	4125	4286	4245	11751	11809	11740	11834
2	4105	4108	4300	4228	11931	11926	11821	11778
3	4094	4104	4329	4234	12152	12135	11950	11769
4	4071	4122	4355	4257	12389	12383	12095	11867
5	4065	4133	4341	4265	12554	12518	12177	11933
6	4053	4130	4318	4260	12631	12548	12215	11953
7	4052	4121	4322	4263	12634	12593	12209	11977
8	4067	4123	4335	4272	12665	12661	12254	12011
9	4079	4145	4327	4274	12724	12661	12340	12044
10	4099	4160	4320	4271	12764	12622	12400	12050
Av.:	4081	4127	4323	4257	12420	12385	12120	11922
CV:	0.58%	0.40%	0.45%	0.38%	2.86%	2.55%	1.80%	0.88%

Av. Fb: 4197 **Av. FU:** 12212
CV: 2.68% **CV:** 1.93%

31 Days Later

Av. FU-Fb:	8015
CF (ng/FU):	6.24E-04
Mt (ng):	0.31
Av. FU-Fb:	8095
CF (ng/FU):	6.18E-04
Mt:	0.31

Summary: Lambda-based Optical Calibration

		CF (ng/FU)	Mt (ng@500 FU)
PCR-based Calibration	Standard Curve:	6.46E-04	0.32
	Ct to Fo Conversion:	6.49E-04	0.32
Direct Calibration	Rep1:	6.18E-04	0.31
	Rep2:	6.24E-04	0.31
	Average:	6.34E-04	0.32
	CV:	2.48%	1.81%

Correlation with direct calibration verifies the precision of both the serial dilution and amplification efficiency estimate

However, the “trueness” of quantitative scale is dependent on the accuracy of the lambda standard quantification

Verification of the “absolute accuracy” of quantitative scale is possible via Limiting Dilution Assay (LDA) which is independent of a quantified standard

Limiting Dilution Assay (LDA)

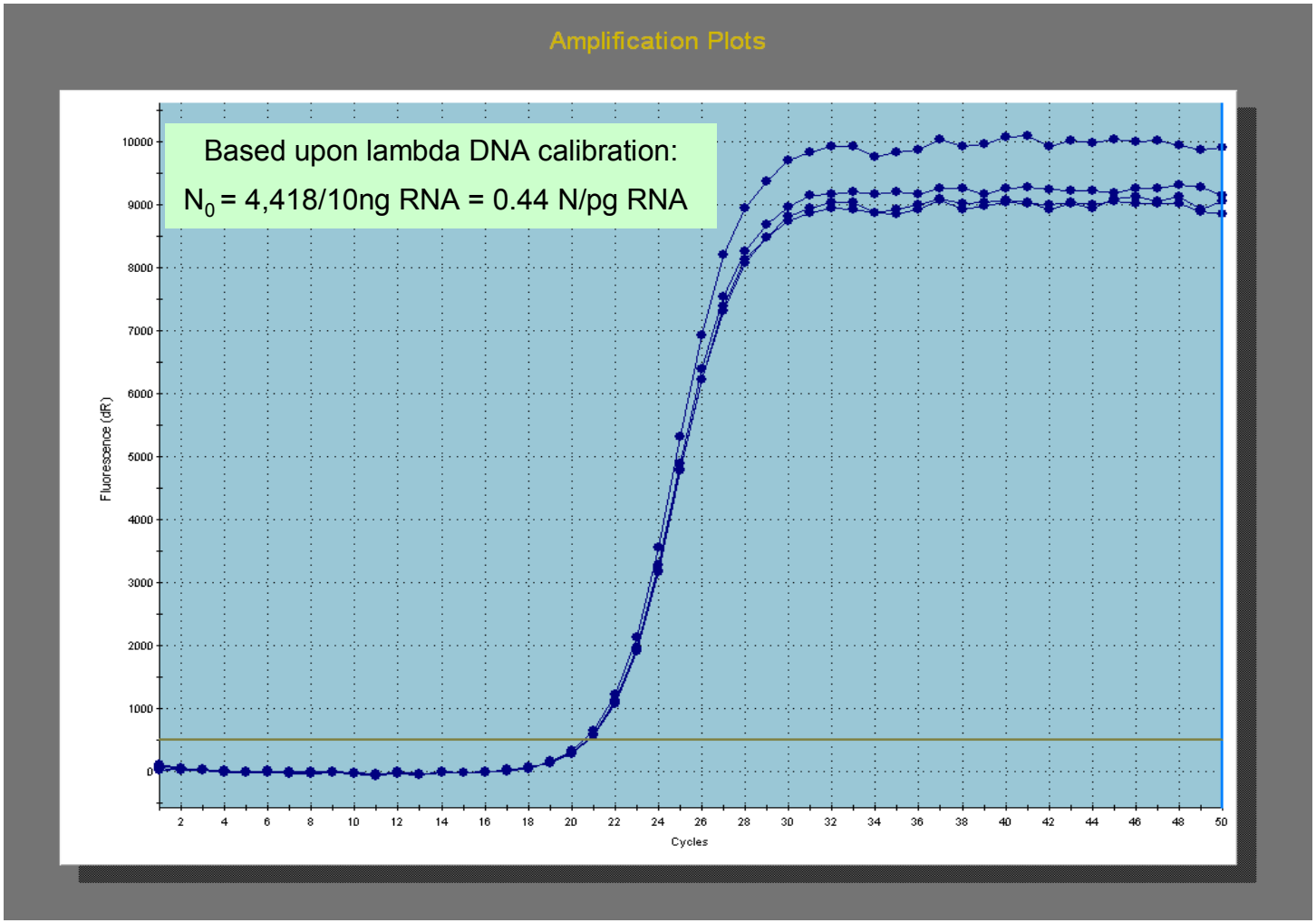
Target Quantity = -Ln(Failures/Total) Wang and Spadoro (1998)

Poisson Distribution at Low Concentration

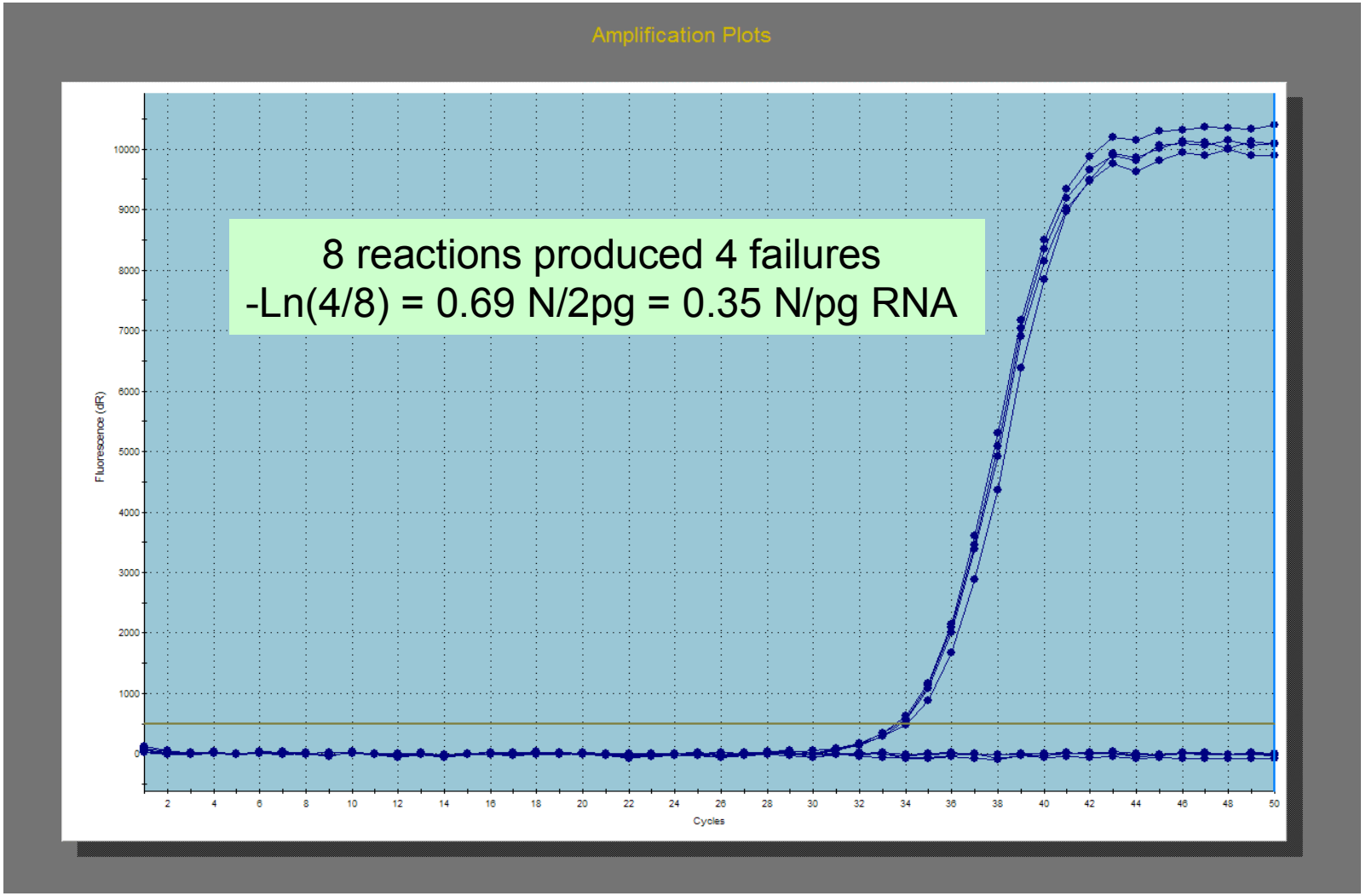
	Actual N delivered					
N per Vol	0	1	2	3	4	5
0.25	77.88%	19.47%	2.43%	0.20%	0.01%	0.00%
0.5	60.65%	30.33%	7.58%	1.26%	0.16%	0.02%
1	36.79%	36.79%	18.39%	6.13%	1.53%	0.31%
2	13.53%	27.07%	27.07%	18.04%	9.02%	3.61%
3	4.98%	14.94%	22.40%	22.40%	16.80%	10.08%
4	1.83%	7.33%	14.65%	19.54%	19.54%	15.63%
5	0.67%	3.37%	8.42%	14.04%	17.55%	17.55%
6	0.25%	1.49%	4.46%	8.92%	13.39%	16.06%
7	0.09%	0.64%	2.23%	5.21%	9.12%	12.77%
8	0.03%	0.27%	1.07%	2.86%	5.73%	9.16%
9	0.01%	0.11%	0.50%	1.50%	3.37%	6.07%
10	0.00%	0.05%	0.23%	0.76%	1.89%	3.78%

2-Step RT-qPCR

Arabidopsis Seedling cDNA: PHABULOSA (HB-zip)



*cDNA diluted to 2 pg RNA per reaction, ~0.88 **PHB** cDNA molecules, X8 reactions*



Validation via Limiting Dilution Assay (LDA)

Target Quantity = -Ln(Failures/Total) Wang and Spadoro (1998)

cDNA (pg RNA) per Rxn	Total # of Rxns	# of Failures	PHB cDNA Molecules per Rxn	PHB cDNA Molecules per pg RNA
2.0	8	4	0.69	0.35
4.5	48	12	1.39	0.31
6.0	40	2	3.00	0.50
6.0	36	3	2.48	0.41
			Average:	**0.39**
			CV:	21.4%

Predicted (lambda-based scale) = **0.44** N/pg RNA

Limiting dilution assays verifies the “absolute accuracy” of the lambda-based quantitative scale as applied to PCR amplification of a cDNA template

Factors Impacting Reaction Fluorescence

- SYBR Green I fluorescence is temperature dependent
- Enzyme formulation impacts SYBR Green I fluorescence
- Tubes: batch to batch variation (although possibly rare)
- Reaction volume (although small, evident for fold differences)
- ROX normalization not recommended (nor necessary)