Protein detection & quantification

- **Spectrophotometric Determination of Protein Concentration**
- **Lowry, Bradford, BCA – what are the differences?**
- **Silver staining and related techniques**
- **Quantitation of Coomassie gels**
- **Specific detection with antibodies (“Western blots”)**
- **(Semi-)Quantification of Western blots (“Densitometry”) and the dynamic range problem**
- **Radiolabelling of proteins, X-ray films & phosphoimager plates**

Information about enzymes needed for cloning

- The New England Biolabs (NEB) catalog
  https://www.neb.com/tools-and-resources
- ReBase (Restriction Enzyme Database)
  http://rebase.neb.com/rebase/rebase.html
- Calculating enzyme amounts?
  http://mcblserver.ltdk.helsinki.fi/recalc/*

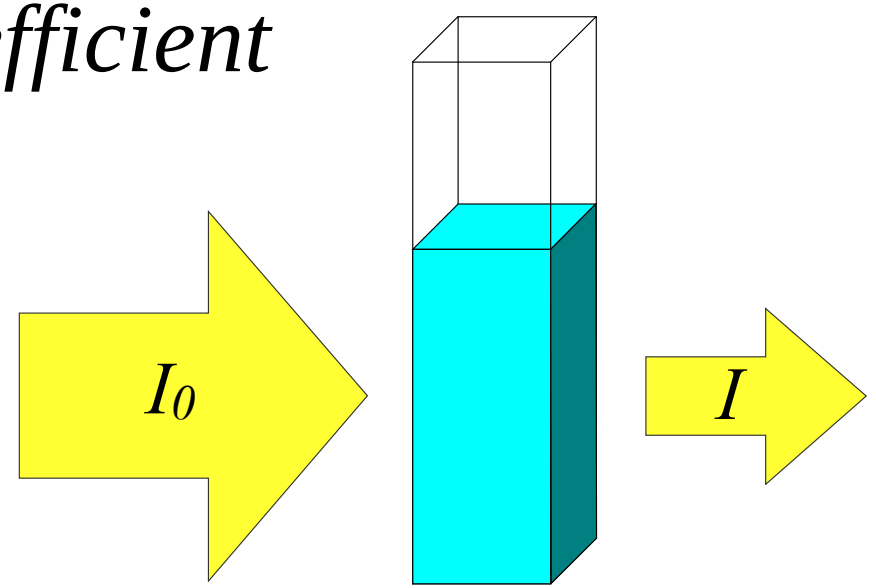
*Account needed! Anybody php/mysql?

Beer-Lambert Law

$$A = \varepsilon \cdot l \cdot c$$

A absorption
ε molar extenction coefficient
l length
c concentration

$$A = \log \frac{I_0}{I}$$



Fluorescence of amino acids and proteins

	Max. Absorption (nm)	Emission (nm) (after lifetime of ~ 2-7 ns)
Tryptophan	280	348
Tyrosine	274	303
Phenylalanine	257	282
EGFP	488	509

The theory is clear, but the paracticalities are confusing...

mjeltsch — more — 80×24

more

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PEPSTATS of std-hVEGF-C from 1 to 121

Molecular weight = 13606.48             Residues = 121
Average Residue Weight  = 112.450       Charge   = 5.0
Isoelectric Point = 7.7305
A280 Molar Extinction Coefficient  = 9530
A280 Extinction Coefficient 1mg/ml = 0.70
Improbability of expression in inclusion bodies = 0.794

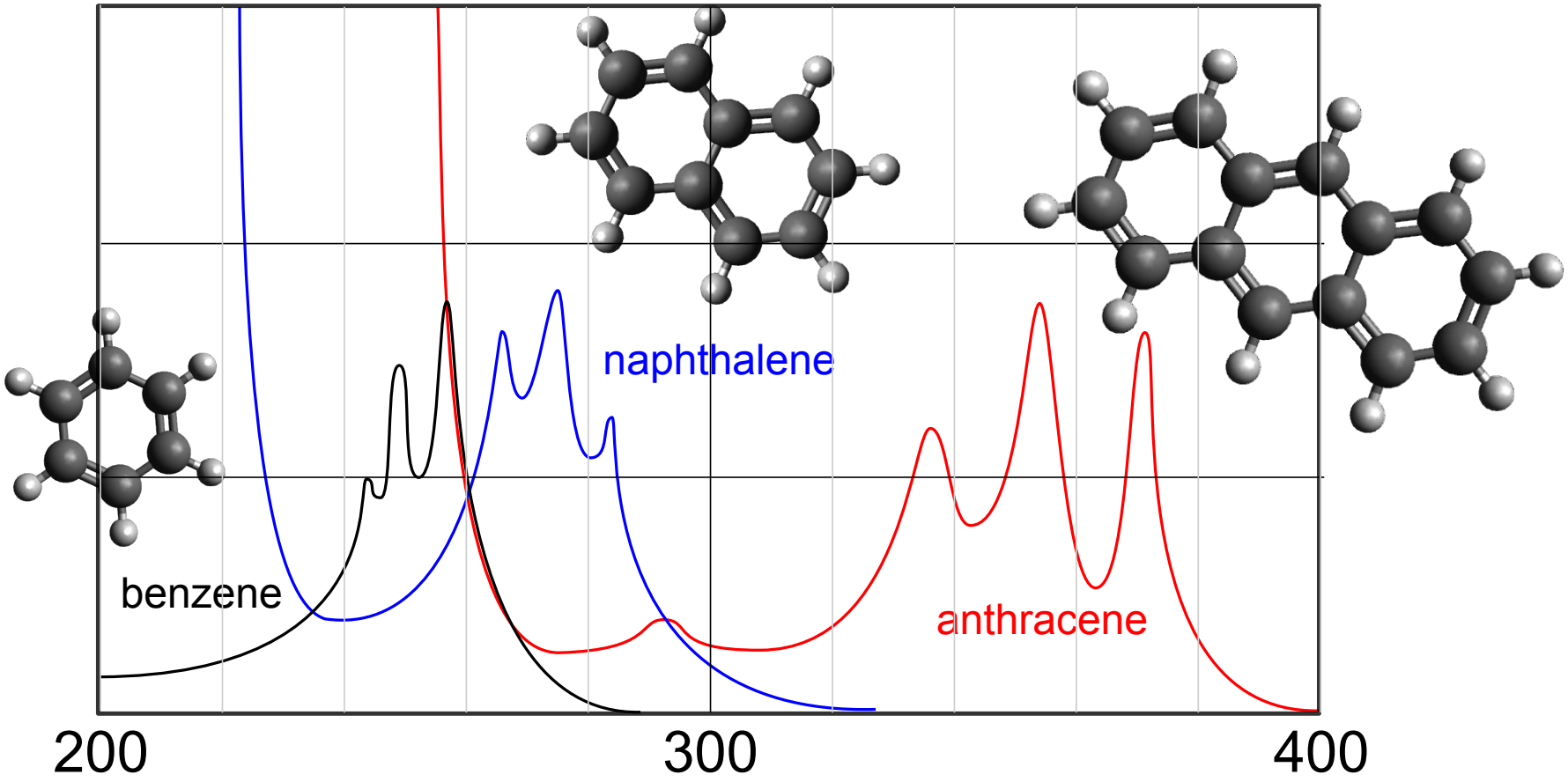
Residue         Number          Mole%           DayhoffStat
A = Ala         5               4.132           0.480
B = Asx         0               0.000           0.000
C = Cys         9               7.438           2.565
D = Asp         3               2.479           0.451
E = Glu         8               6.612           1.102
F = Phe         6               4.959           1.377
G = Gly         6               4.959           0.590
H = His         8               6.612           3.306
I = Ile         6               4.959           1.102
J = ---         0               0.000           0.000
K = Lys         8               6.612           1.002
L = Leu         6               4.959           0.670
M = Met         3               2.479           1.458
std-hvegf-c.pepstats
```

	unit	
A280 Molar Extinction Coefficient	$M^{-1}cm^{-1}$	SI units
A280 Extinction Coefficient 1mg/ml ("optical absorbance at 1g/l")	cm^2mg^{-1}	most common
A280 Percent Solution Absorbance (E1%)	$0.1 \cdot cm^2mg^{-1}$	rarely used

Calculating ε (280nm)

$$\varepsilon 280nm = (5500 n\mathrm{Trp}) + (1490 n\mathrm{Tyr}) + (125 n\mathrm{S\text{-}S})$$

Why does Phe not absorb at 280nm? It is, after all, also an aromatic ring with delocalized Π-electrons. It does absorb UV, but it does so at shorter wave lengths, because its congugated system is small.



ε or cm^2mg^{-1}?

$$c = \frac{A}{\varepsilon \cdot l}$$

Example calculation:

BSA, ε=0.667 cm^2mg^{-1}, l = 1 cm, A = 1.334

$$c = \frac{unitless!}{cm^2\, mg^{-1} \cdot cm} = \frac{mg}{cm^3}$$

$$c = \frac{1.334}{0.667\, cm^2\, mg^{-1} \cdot 1\, cm} = 2\,\frac{mg}{cm^3}$$

multiply by MW (g/mol)

BSA, ε=43824 $M^{-1}cm^{-1}$, l = 1 cm, A = 1.334

$$c = \frac{unitless!}{M^{-1}\, cm^{-1} \cdot cm} = M$$

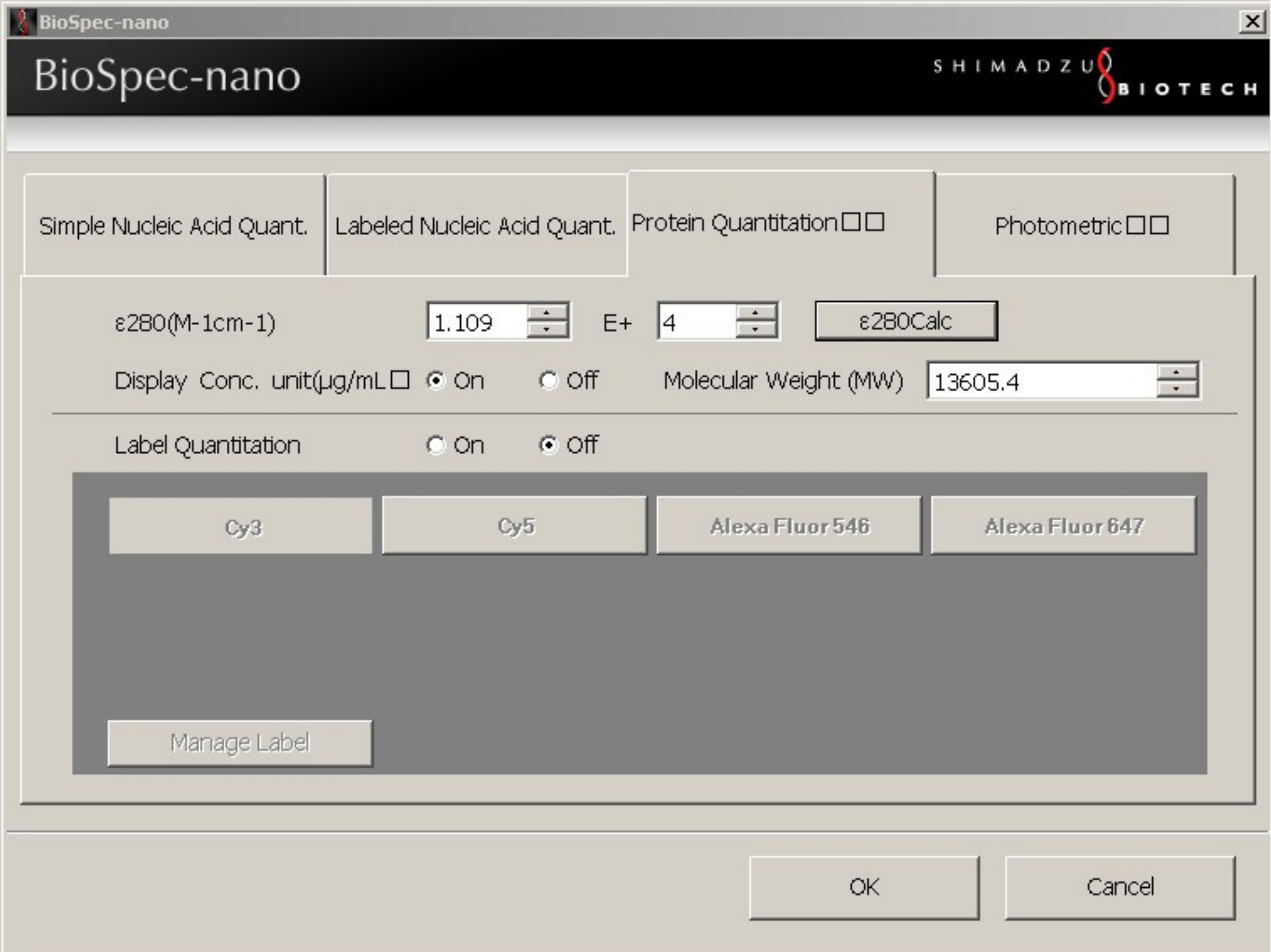
$$c = \frac{1.334}{43824\, M^{-1}\, cm^{-1} \cdot 1\, cm} \sim 3 \cdot 10^{-5}\, M$$

BSA: 66463 g/mol

Less than 5% of the photons make it through the cuvette!

Converting from mg/ml to mol/l

	MW (g/mol)	*g/l (or mg/ml)*	*%*	*mol/l (molarity, M)*
		÷10 →		
			÷MW →	
BSA	66433	1	0.1	$15 \cdot 10^{-6}$ (= 15 µM)
BSA		100	10	$1.5 \cdot 10^{-3}$ (= 1.5 mM)
IgG	~150000	1	0.1	~6.7 µM
RNaseA	~13700	1	0.1	73 µM


BioSpec-nano
BioSpec-nano
SHIMADZU BIOTECH
Simple Nucleic Acid Quant.
Labeled Nucleic Acid Quant.
Protein Quantitation
Photometric
ε280(M-1cm-1)
1.109
E+
4
ε280Calc
Display Conc. unit(μg/mL
On
Off
Molecular Weight (MW)
13605.4
Label Quantitation
On
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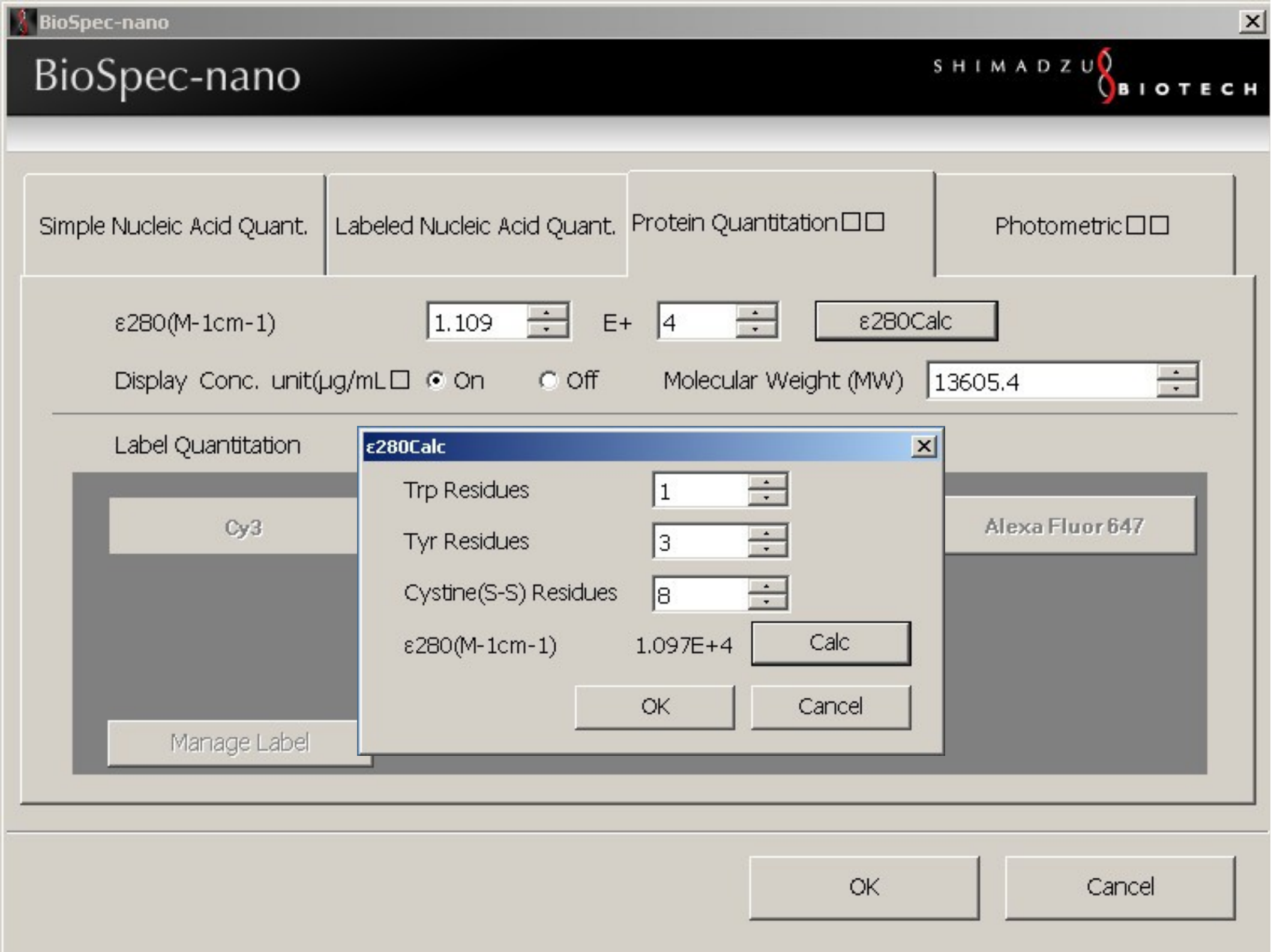
Cy3
Cy5
Alexa Fluor 546
Alexa Fluor 647
Manage Label
OK
Cancel


BioSpec-nano
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SHIMADZU BIOTECH
Simple Nucleic Acid Quant.
Labeled Nucleic Acid Quant.
Protein Quantitation
Photometric
ε280(M-1cm-1)
1.109
E+
4
ε280Calc
Display Conc. unit(μg/mL)
On
Off
Molecular Weight (MW)
13605.4
Label Quantitation
Cy3
Alexa Fluor 647
Manage Label
ε280Calc
Trp Residues
1
Tyr Residues
3
Cystine(S-S) Residues
8
ε280(M-1cm-1)
1.097E+4
Calc
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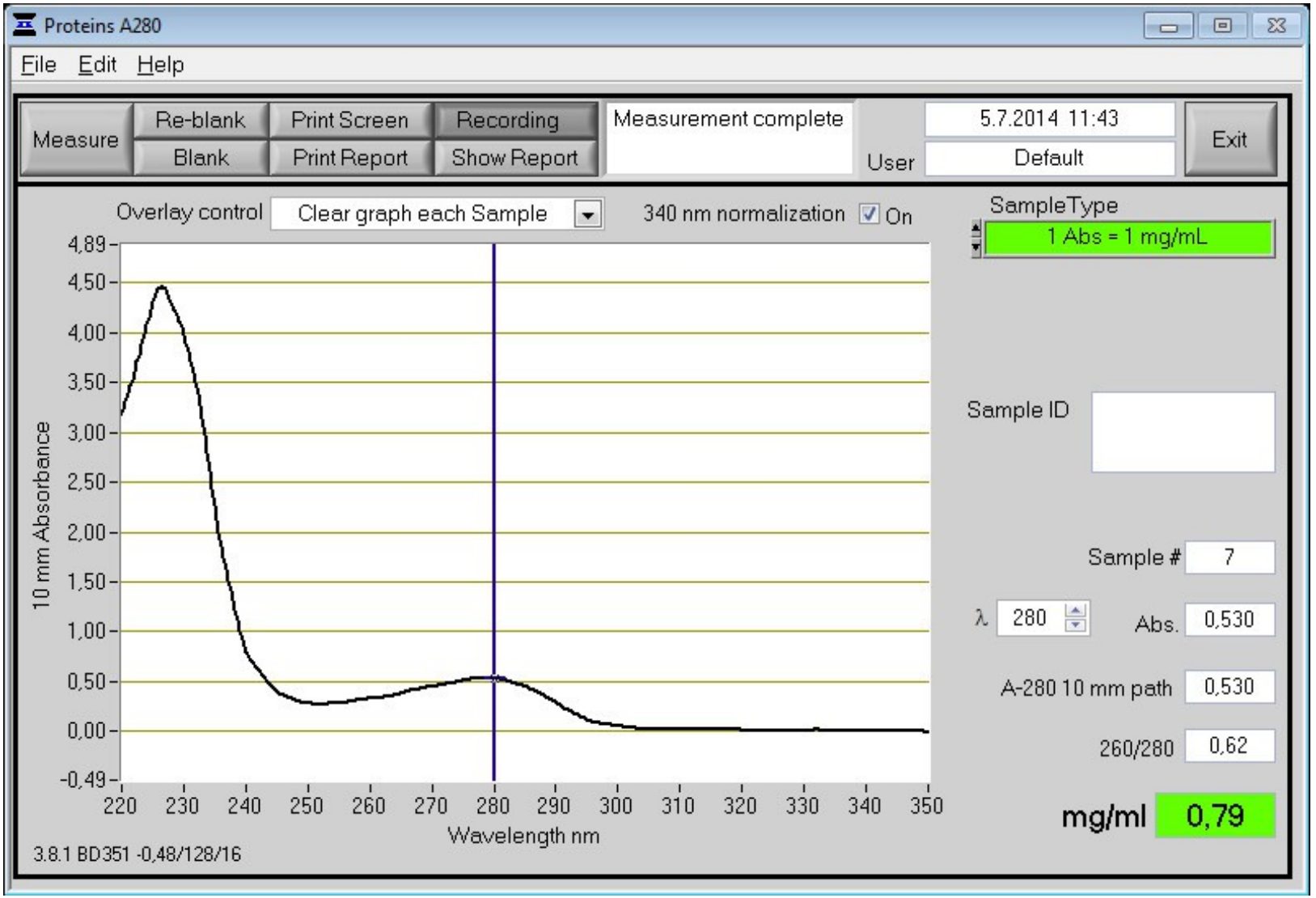
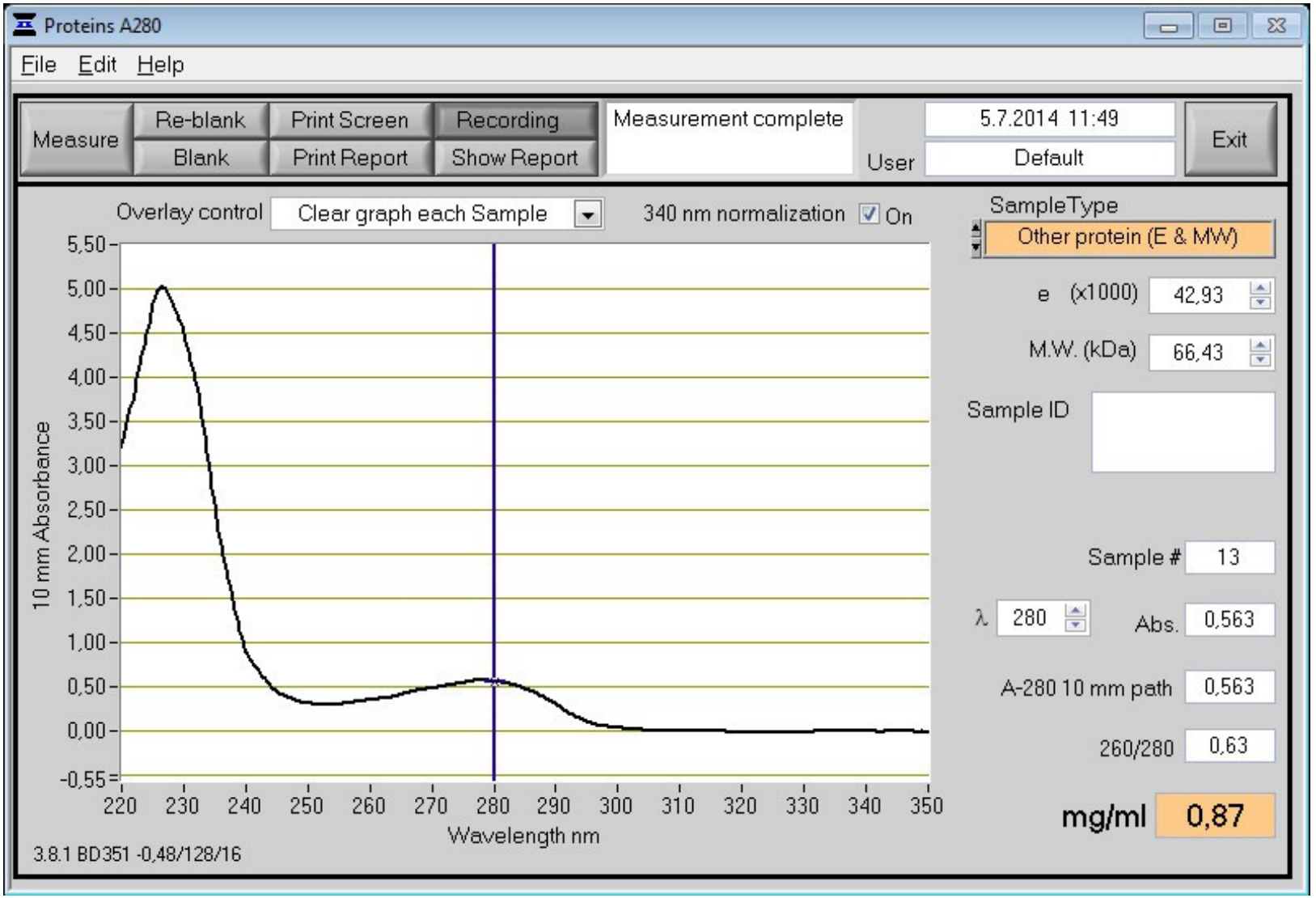
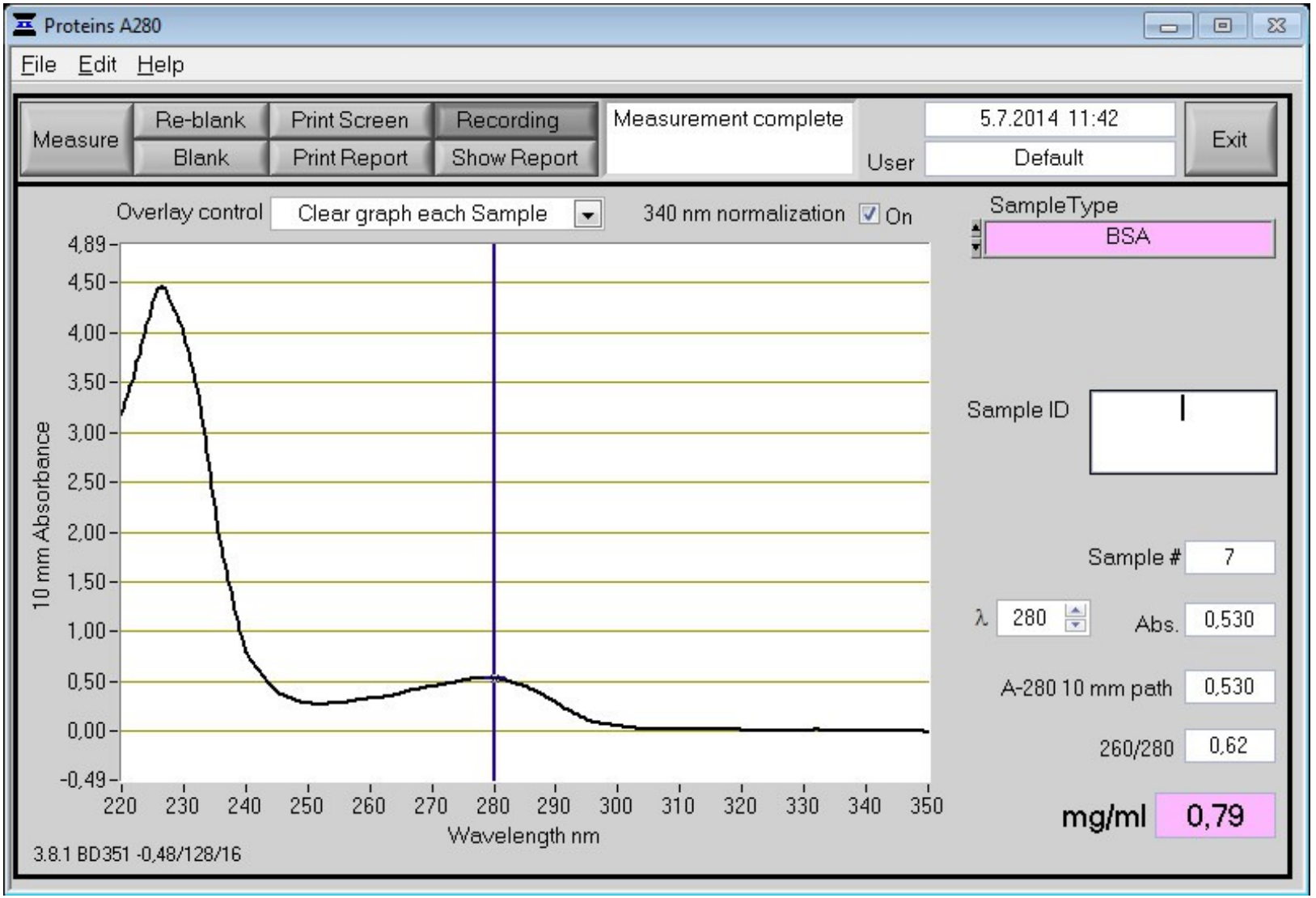
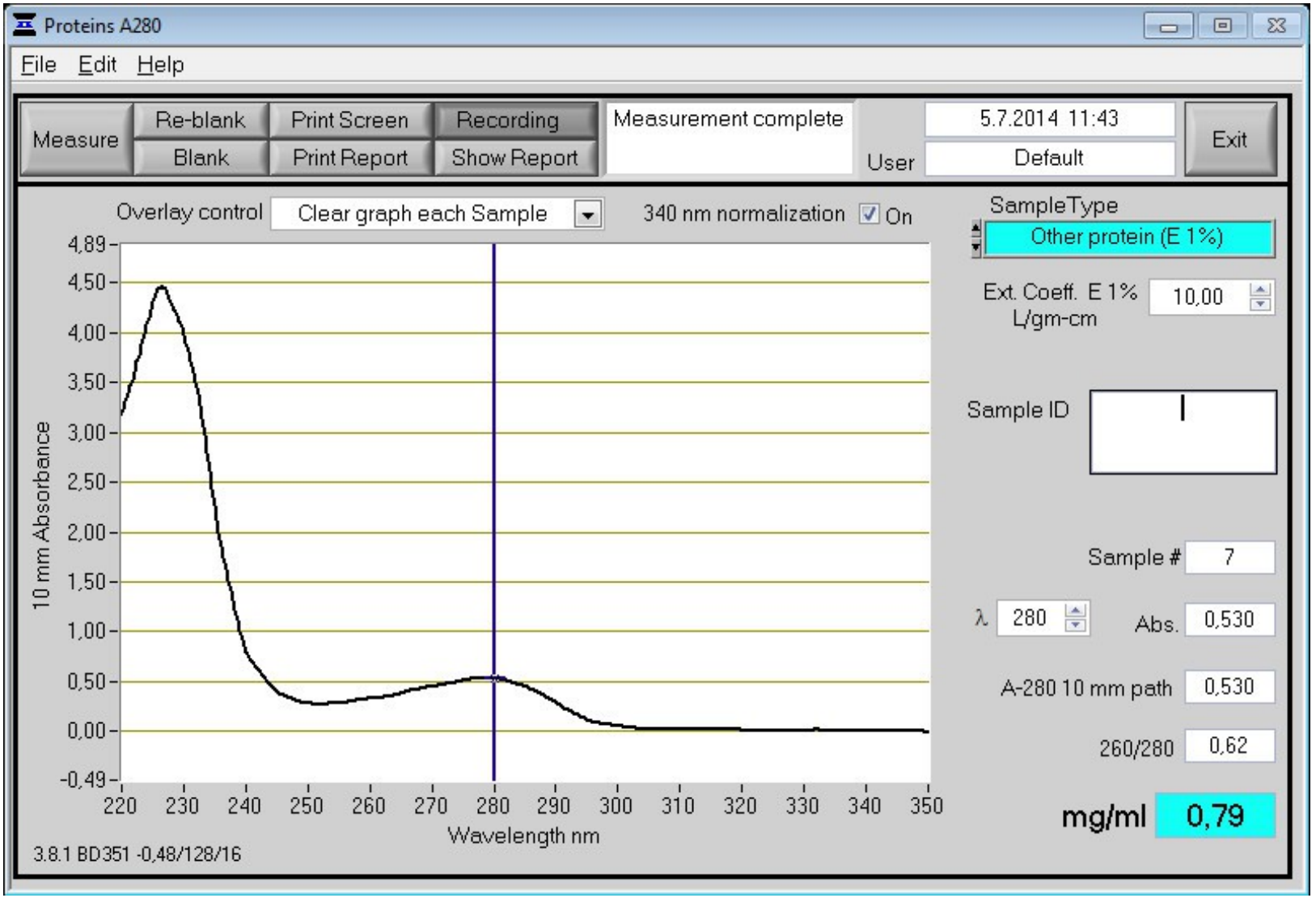






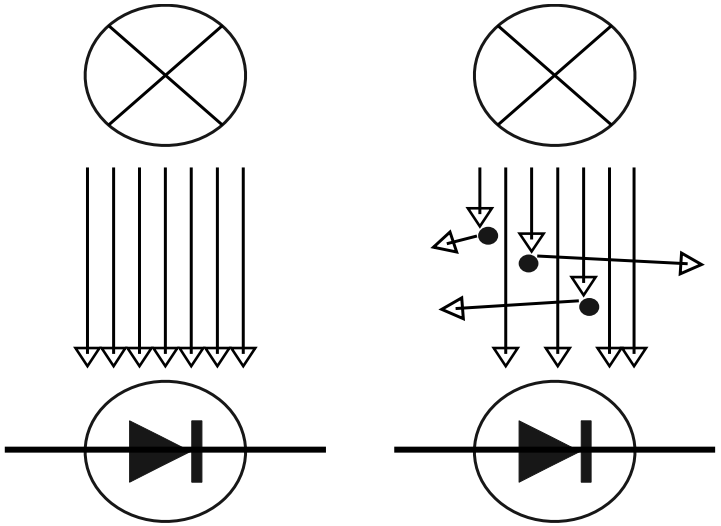


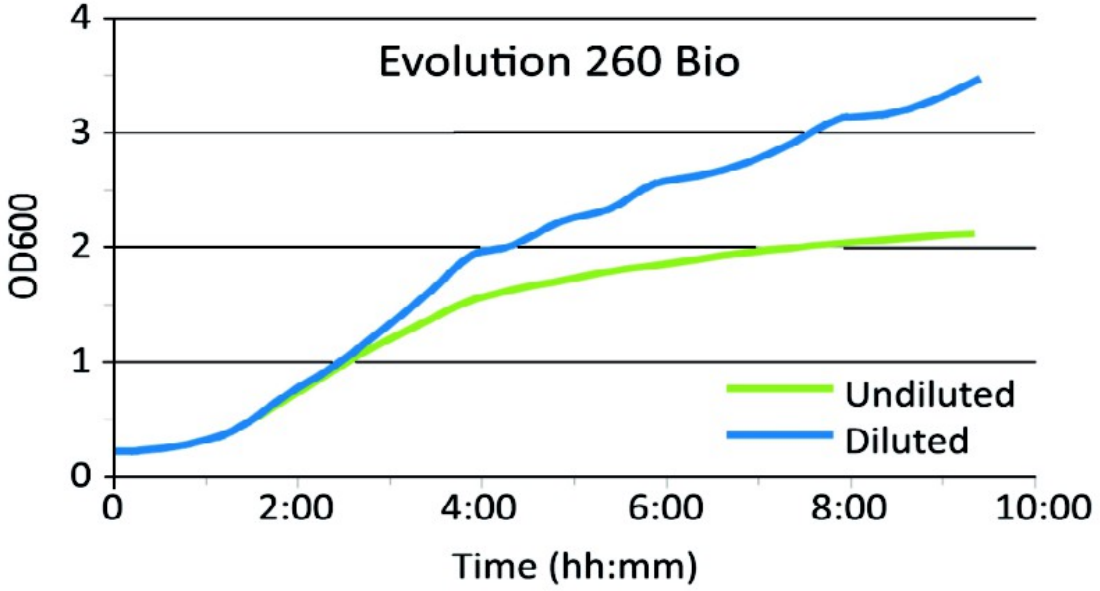
Measuring bacterial growth by OD

Beer-Lambert law $A = \varepsilon \cdot c \cdot l$

Homogenous, true solution (no suspension!), parallel light beams

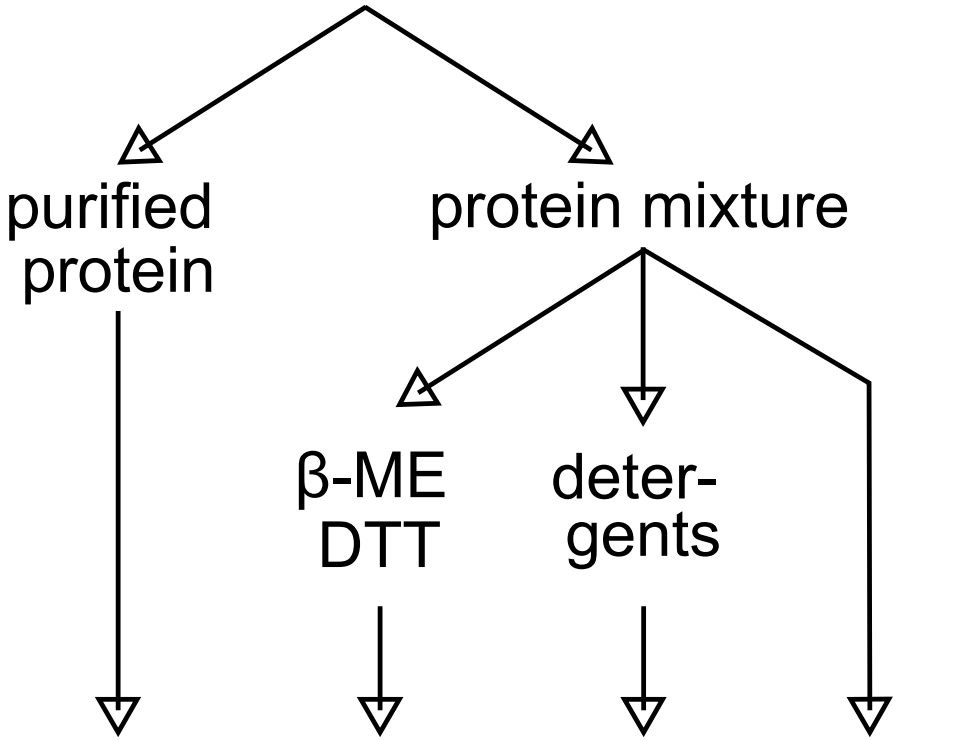
OD600 for measuring bacterial growth does not measure absorption, but scattering
(⇒ huge differences between photometers for bacterial OD600 measurements)





Linear range: OD 0-1

UV, Lowry, Bradford or BCA?

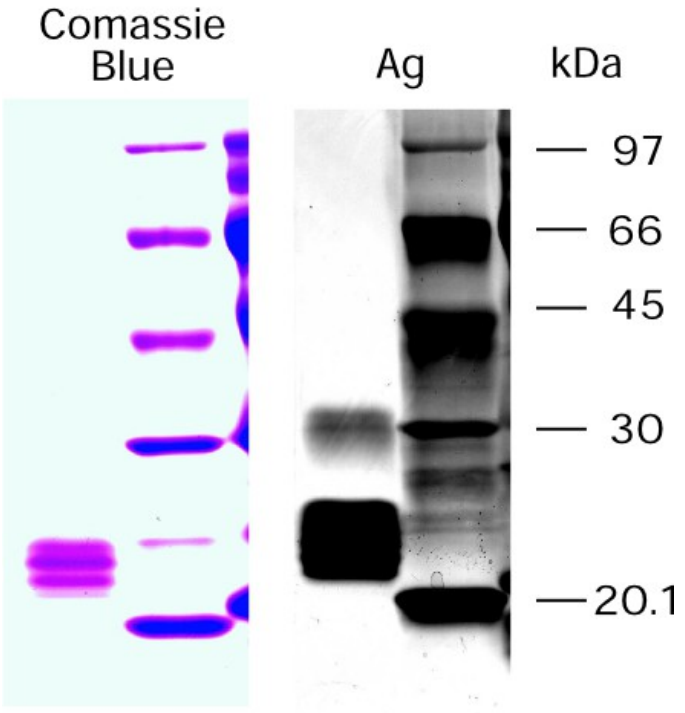


What if no assay is compatible? → TCA precipitation!

Coomassie, silver staining, etc.

- Bradford = Comassie Brilliant Blue G-250

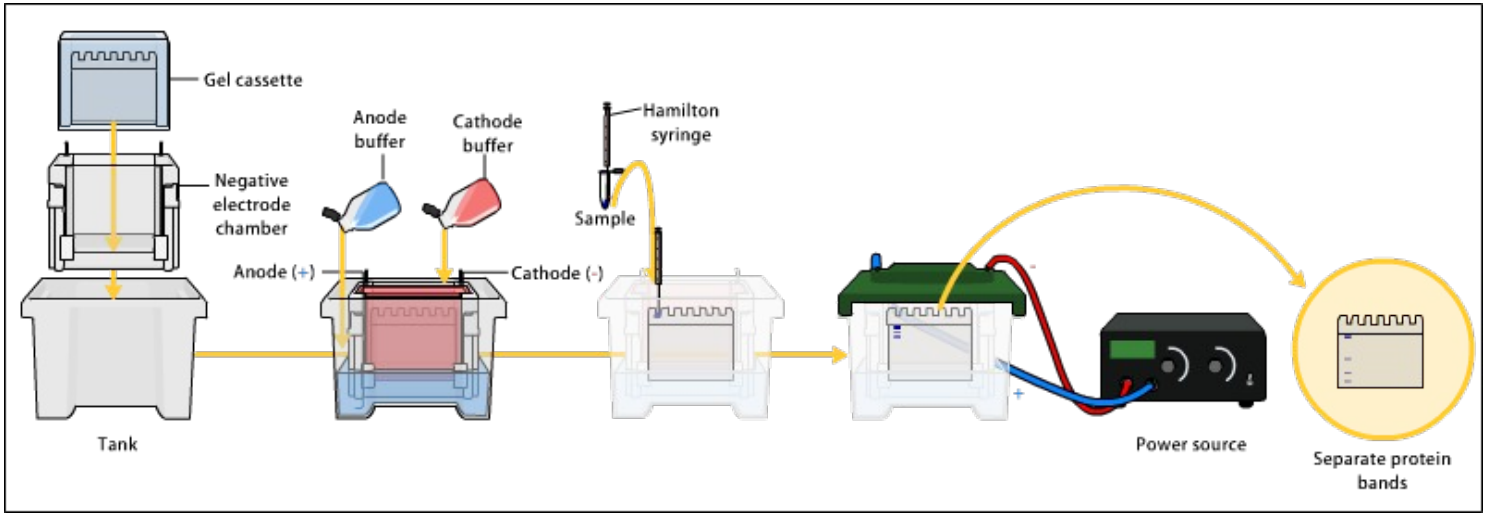
Staining method	*Detecton limit (ng)*	*Quantitation*	*Mass spec*
Silver	0.1-1	no	no*
Comassie	~100	yes	yes
Colloidal Coomassie	~10	yes	yes
Copper	~10	no	yes

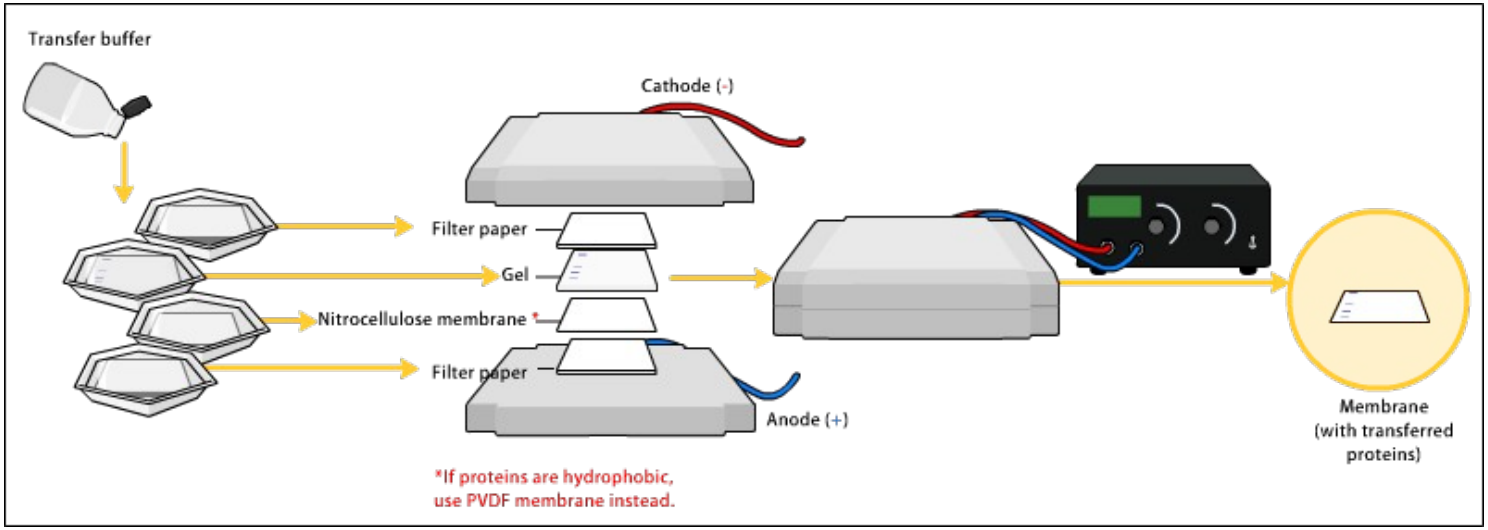


*Special protocols allow mass spec, but they compromise sensitivity

Western Blotting

Detection with specific antibodies after transfer of the proteins to a membrane





Images by Bensaccount

Western Blotting

- Both relative and absolute quantification is possible. Absolute quantification requires a protein standard which is mostly not available.
- Relative quantification is easy with digital capture devices, but tedious with traditional X-ray film.
- Many different commerical solutions exist to replace X-ray film: Syngene's PXi, Li-Cor's Odysee (IR-dyes), Azure's cSeries, etc.
- Digital data aquisition is superior in all respect except for two issues:

  1. Sensitivity is still an issue if you need to detect (non-quantitatively) very low protein amounts! Don't let the massive propaganda machinery of the producers fool you!

  2. Exposure is possible only for one gel at a time as opposed to X-ray film casettes, where as many blots can be exposed simultaneously as there are film casettes

Quantitation of Coomassie gels and scanned Western blots

- http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/
- ImageJ (NIH): http://imagej.nih.gov/ij/
- Not the best, but the standard software, ImageJ2 under development
- ? Icy: http://icy.bioimageanalysis.org/
- ? Jmicrovision: http://www.jmicrovision.com/
- http://lukemiller.org/index.php/2013/02/analyzing-western-blots-with-image-studio-lite/
- Image Studio Lite: http://www.licor.com/bio/products/software/image_studio_lite/index.html

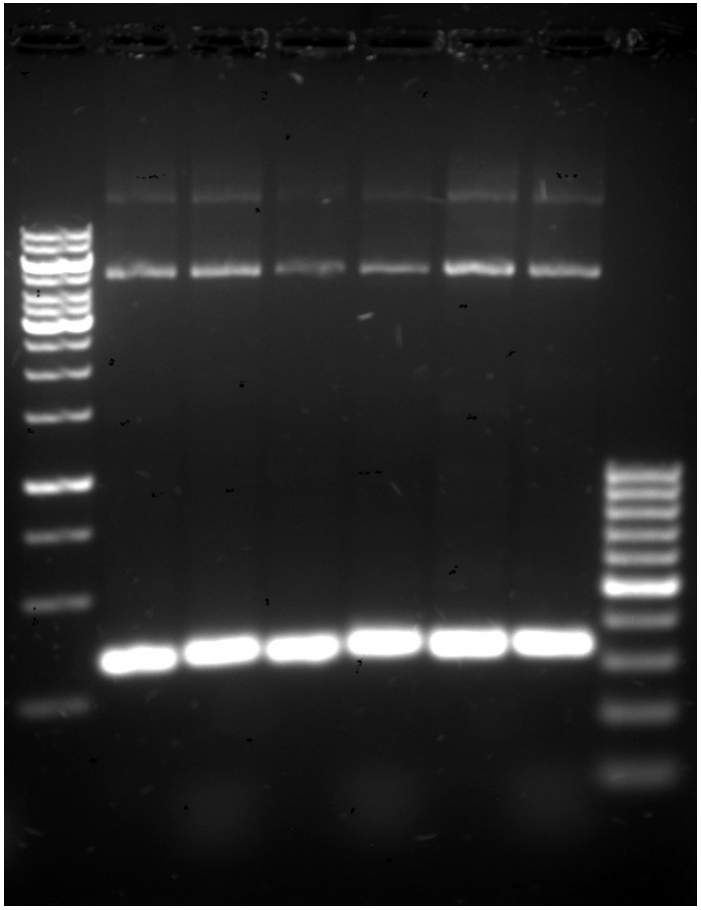
Metabolic labeling

- Growing your cells in cystein- and methionine-deficient medium and adding radioactively labeled cys/met mix (“Easy Tag”, ^{35}S, $t_{1/2}$ ~2.5 months)
- Because all proteins are going to be labeled, you still need a specific reagent: typically you immunoprecipitate your protein with a specific antibody or anti-serum, run a SDS-PAGE and expose the dried gel to X-ray film or phosphoimager plates
- Quantitation as with Western from exposed X-ray film or the digital image
- Works also if regular medium is used (just not as well...)
- Pitfalls: Proteins have different Cys/Met content! The label gets often depleted during the experiment!

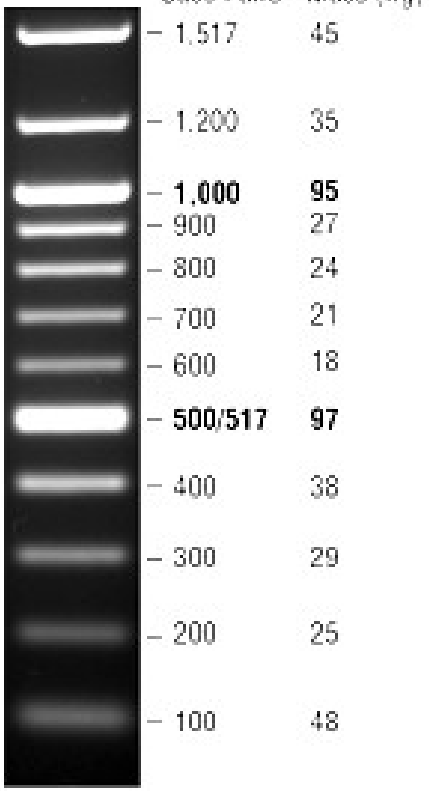
The Cloning Challenge

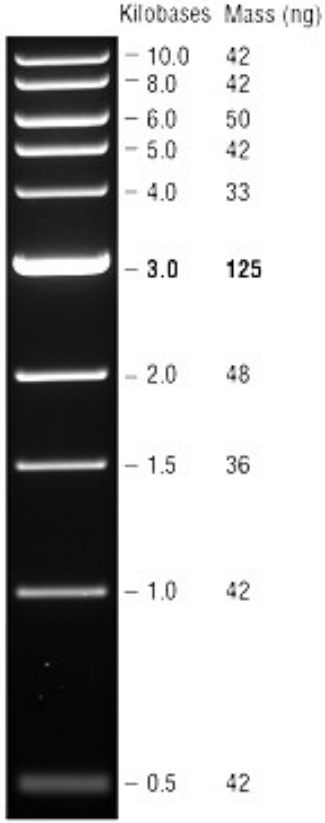
1kb ladder

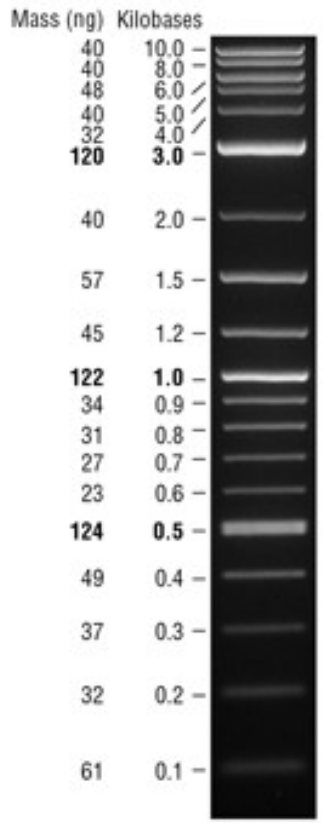
100bp ladder



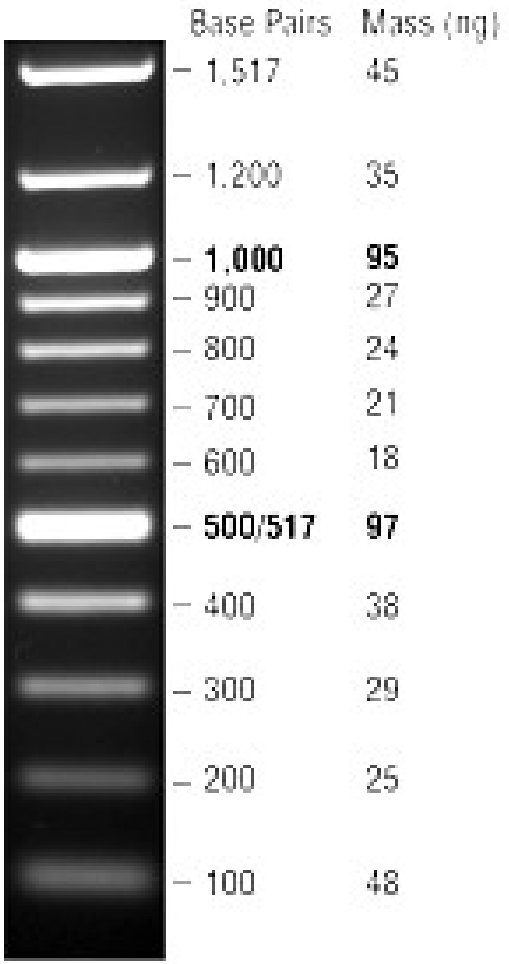
250µg (less than one DNA mass prep) ~200 €







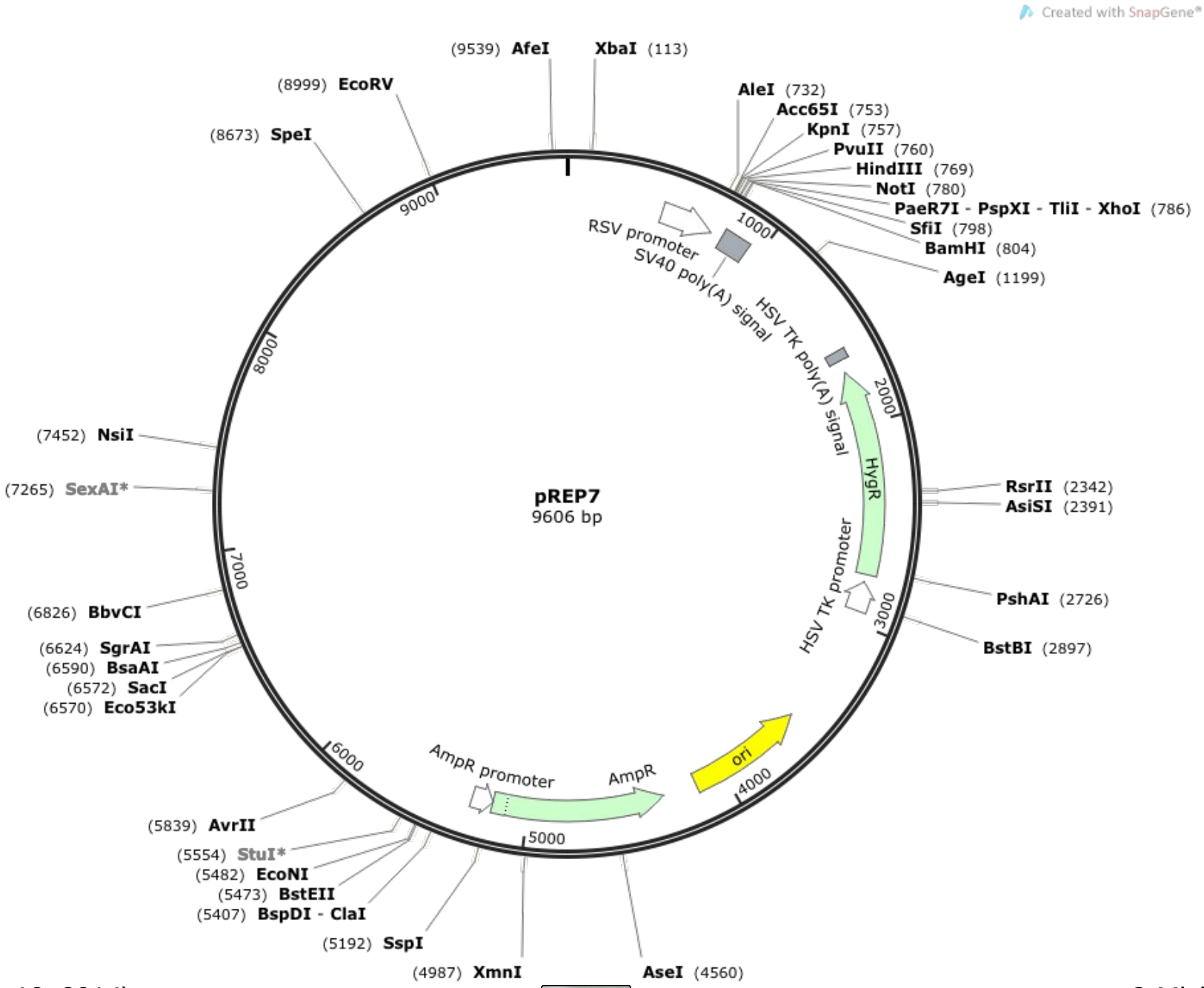
DNA markers



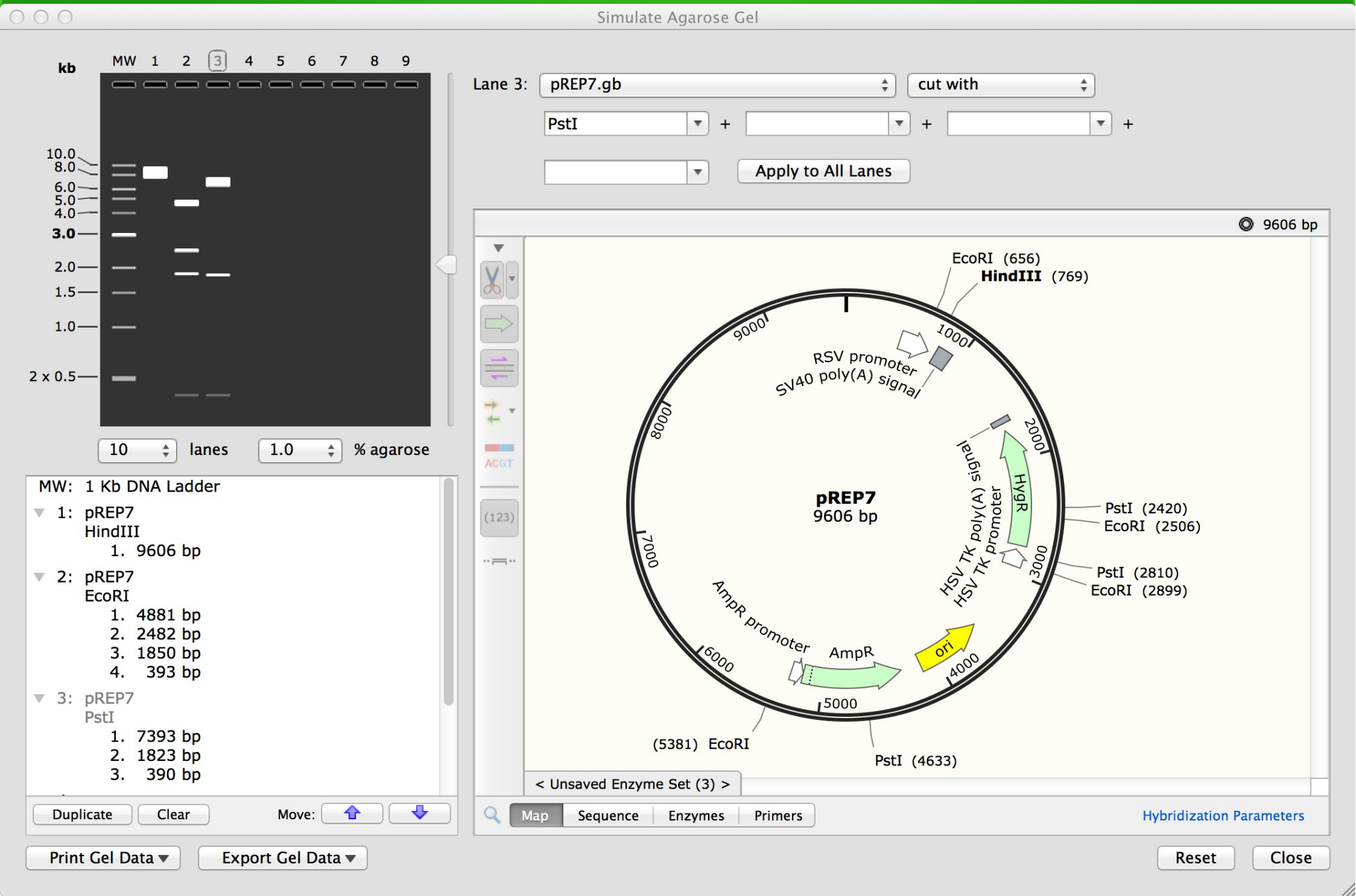
100 bp ladder

λ HIndIII

Example: pREP7



Making a DNA ladder



Making a DNA ladder

Minimize:

- Price: enzymes needed for digesting 2mg DNA (saves about 2k€), getting the construct
- Cloning work & reagents

Maximize:

- Coverage: no big gaps between individual bands, not two bands too close to each other
- Equal band intensities: Only possible to reach by having multiple fragments of the same size