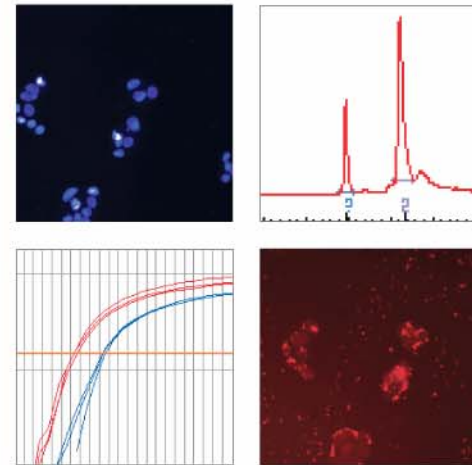


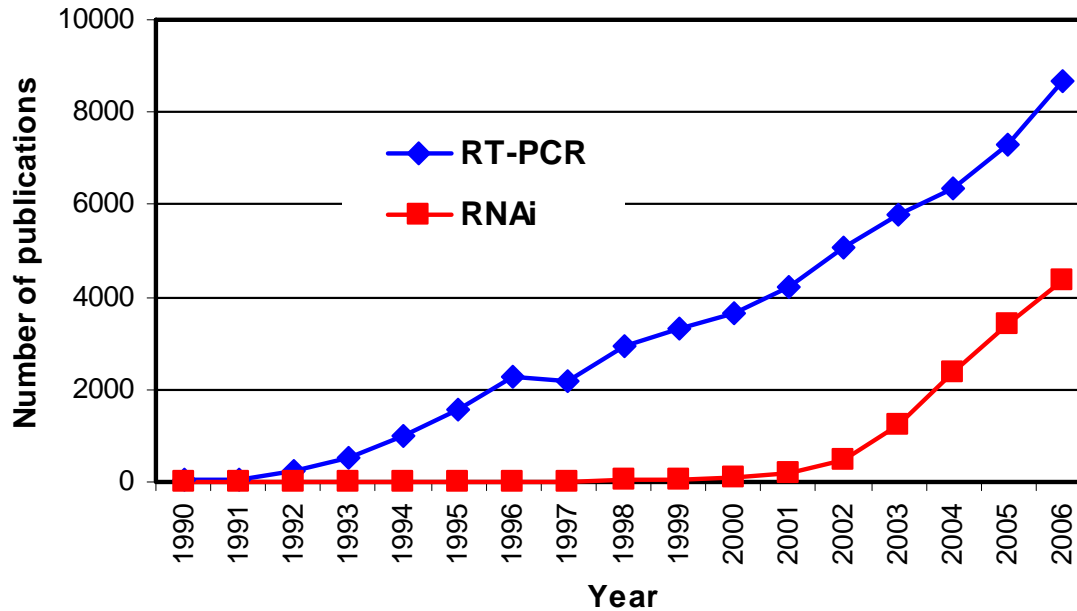
Multi-Discipline Analysis of Gene Silencing: Complementary use of Multiplex RT-qPCR, 2-D electrophoresis and Western blotting for RNAi based pathway analysis.

Eli Hefner, Ph.D.
Sr. Scientist
Gene Expression Division

2007



Comparison of publications with RT-PCR and RNAi



There is a lot of room for growth in the area of RNAi and RT-PCR. It is likely that use of these two technologies will increase in unison.

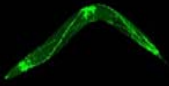
Where did it all begin?



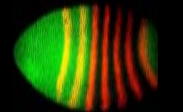
1990 Cosuppression described in petunia



1992 Quelling reported in fungi



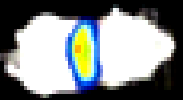
1998 dsRNA identified as causitive agent in *C. elegans*



1998 RNAi used for gene knockout in *Drosophila*



2001 siRNA mediated gene silencing in mammalian cells



2002 Mammalian gene silencing *in vivo*

Dicer

2005 Synthetic dsRNA Dicer substrates enhance RNAi efficacy

Small interfering RNA (siRNA), are produced in vivo when long dsRNA molecules are cleaved into 20-22 base duplexes having 2-base 3' overhangs.

RNAi can be activated by synthetic siRNAs or by longer dsRNA that require Dicer

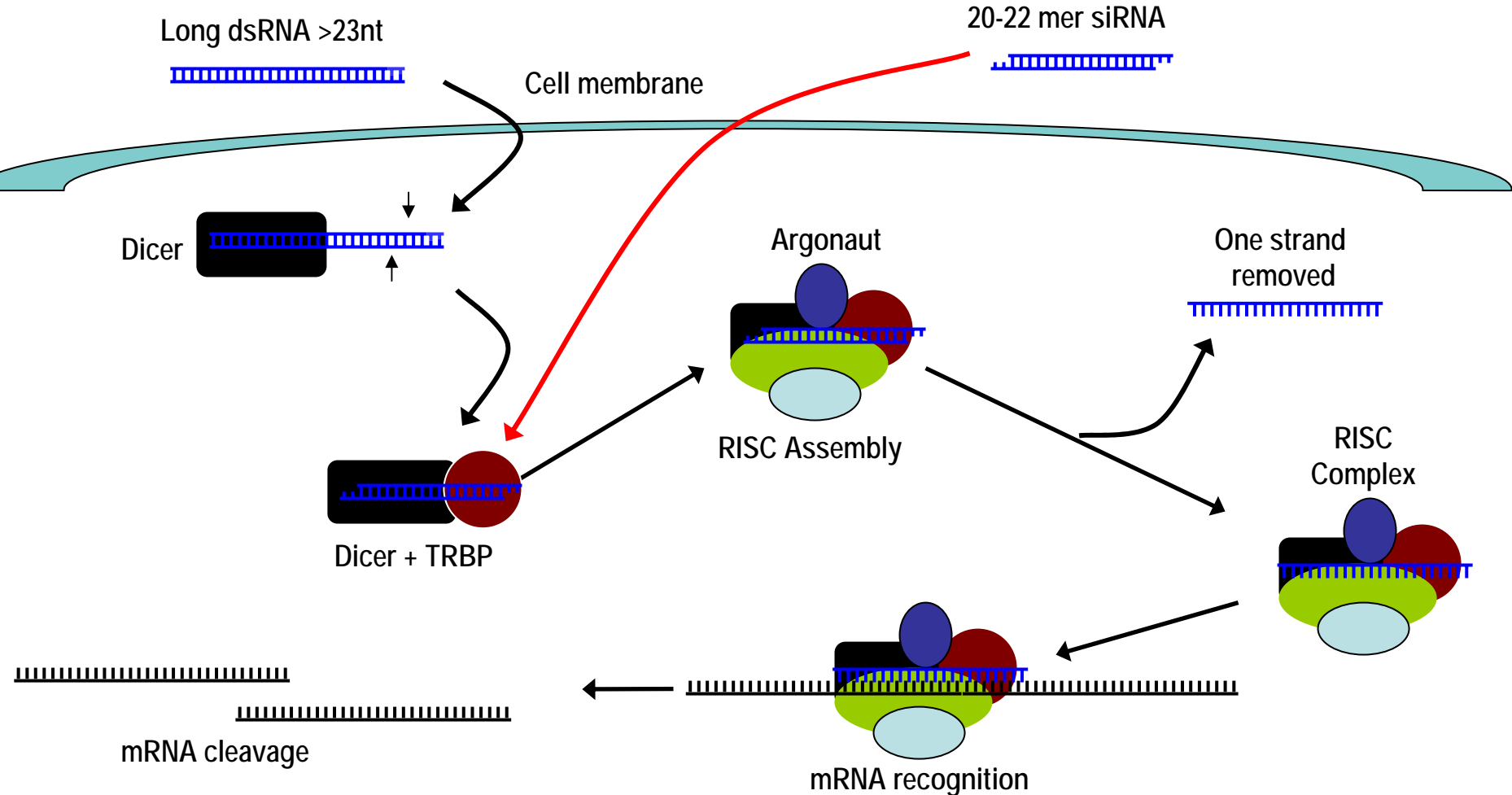


Synthetic siRNAs, 21-23 bases long
with 3' overhangs

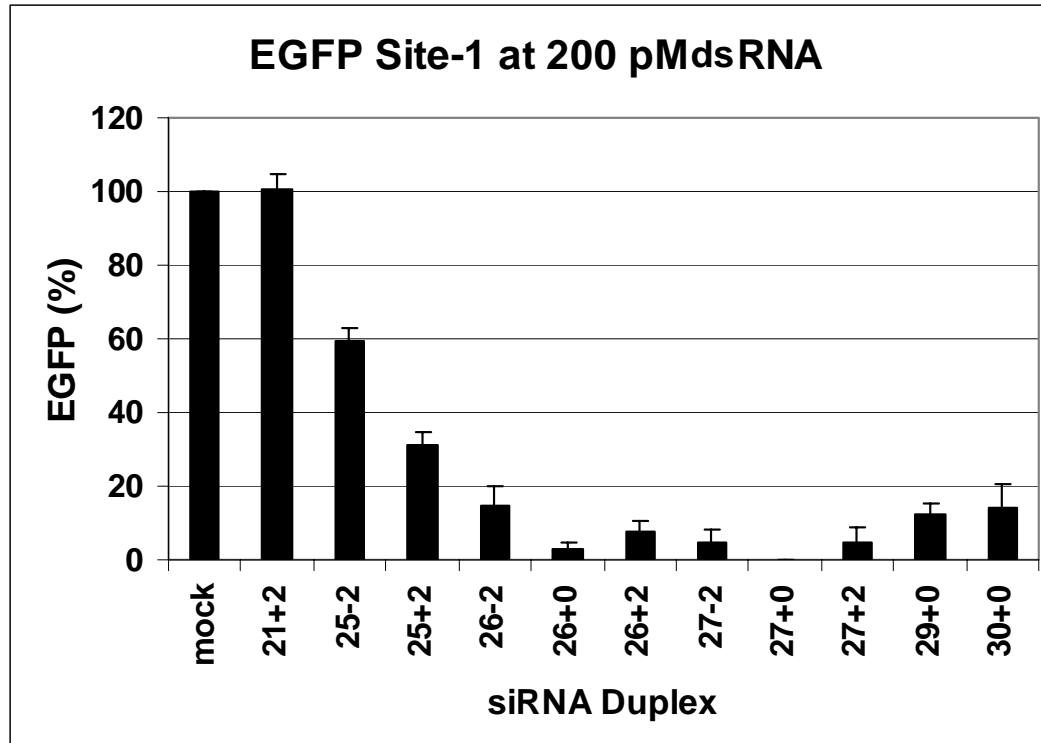


Dicer substrates, >25 bases long
Can have a variety of end structures

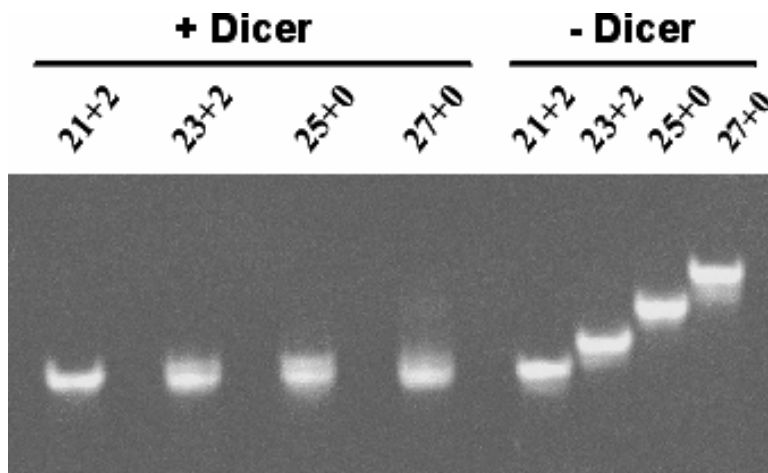
Cellular mechanism of RNAi mediated gene silencing



Increased Potency with Longer Duplexes



Dicer cleaves 23mer and longer duplexes but 21mer duplexes are not a substrate.



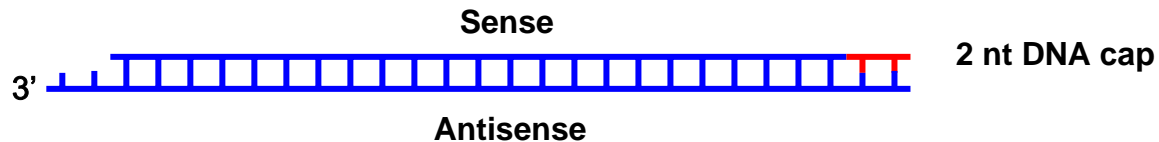
RNA duplexes were incubated overnight with recombinant human Dicer with Mg⁺⁺

RNA duplexes were incubated with recombinant human Dicer with EDTA

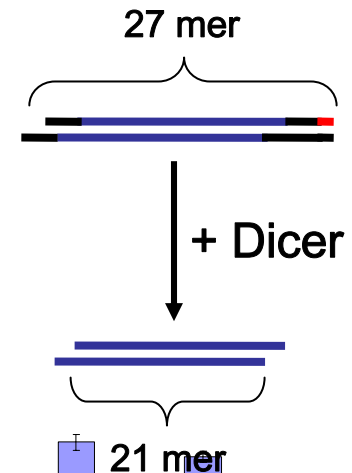
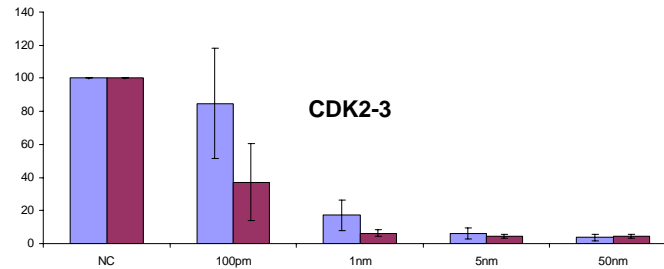
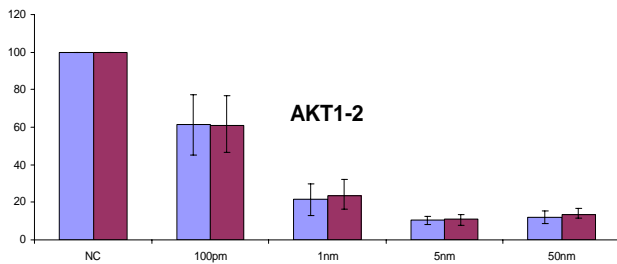
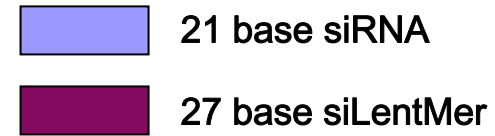


Dicer binds (gel-shifts) longer duplexes better than 21mer

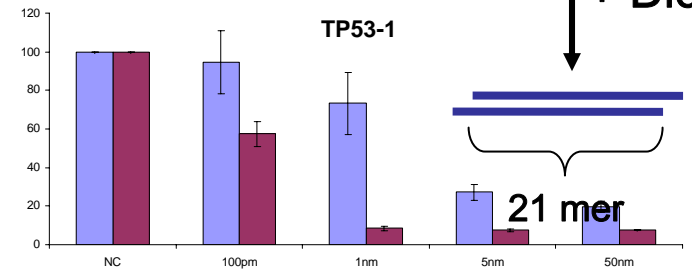
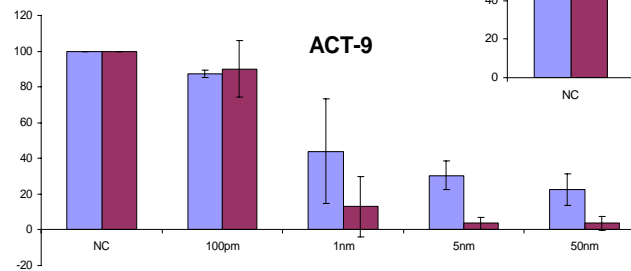
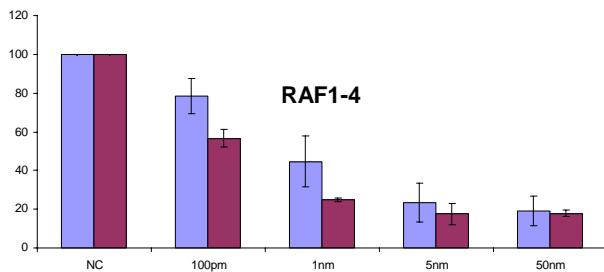
siLentMer Dicer Substrates are 27 nucleotide RNA antisense sequences annealed to a 25 nucleotide RNA / DNA sense sequence.



siLentMers vs siRNAs



Analyzed 5 paired 27 and 21 mer dsRNAs



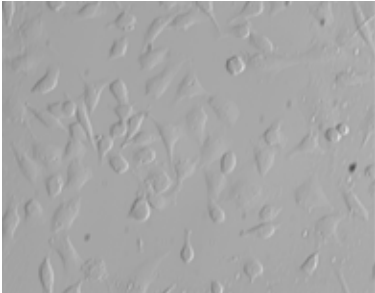
Key considerations when performing RNAi experiments

Transfection efficiency

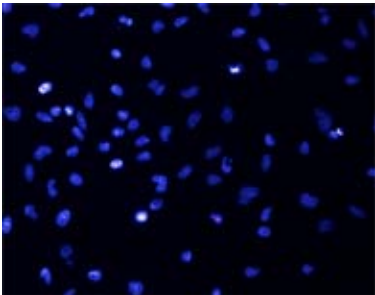
Microscopy

HeLa Cells

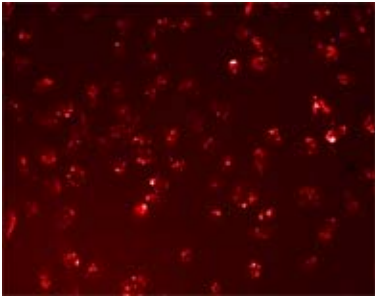
Light
Microscope



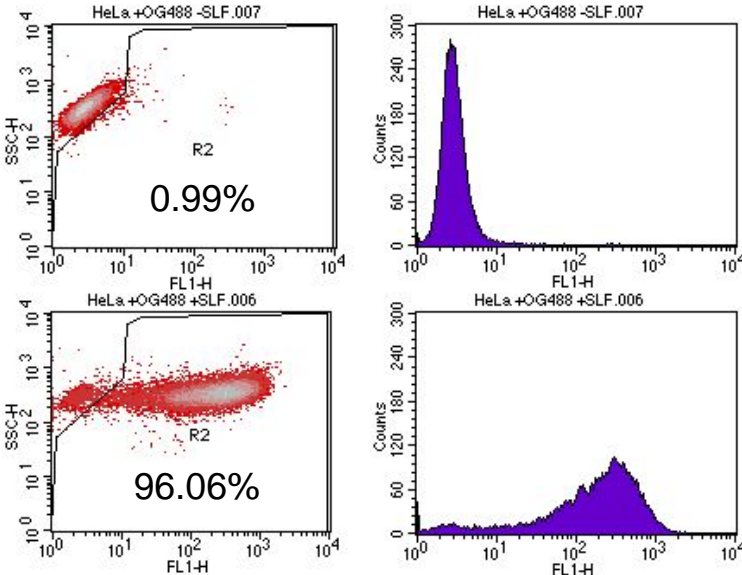
Nuclear
Stain



Fluorescent
Non-silencing
siLentMer

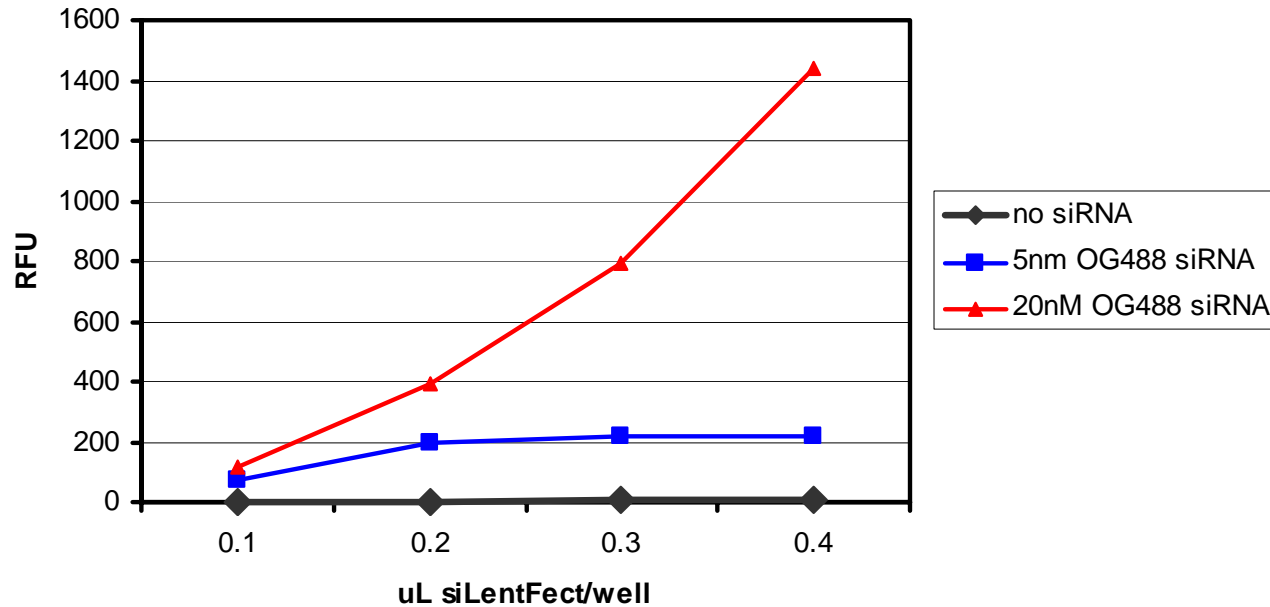


Flow cytometry



Optimizing lipid and dsRNA concentrations

Analysis of transfection efficiency with increasing lipid and dsRNA concentration

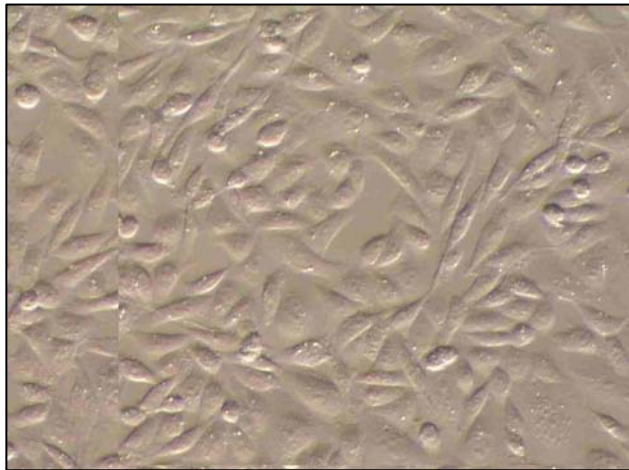


Samples were transfected with a fluorescently labeled dsRNA and a measurement of total fluorescence was taken 24 hours after transfection.

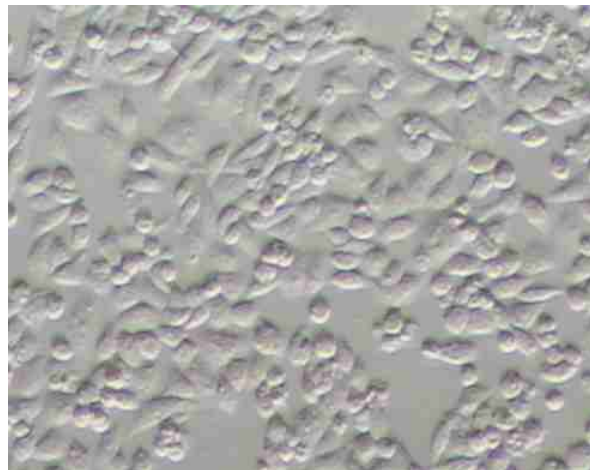
Visual Analysis

- Morphology changes
- Detachment
- Lysis

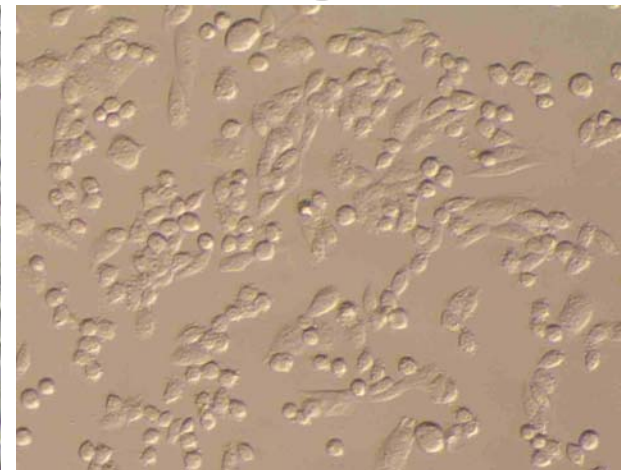
Low



Moderate



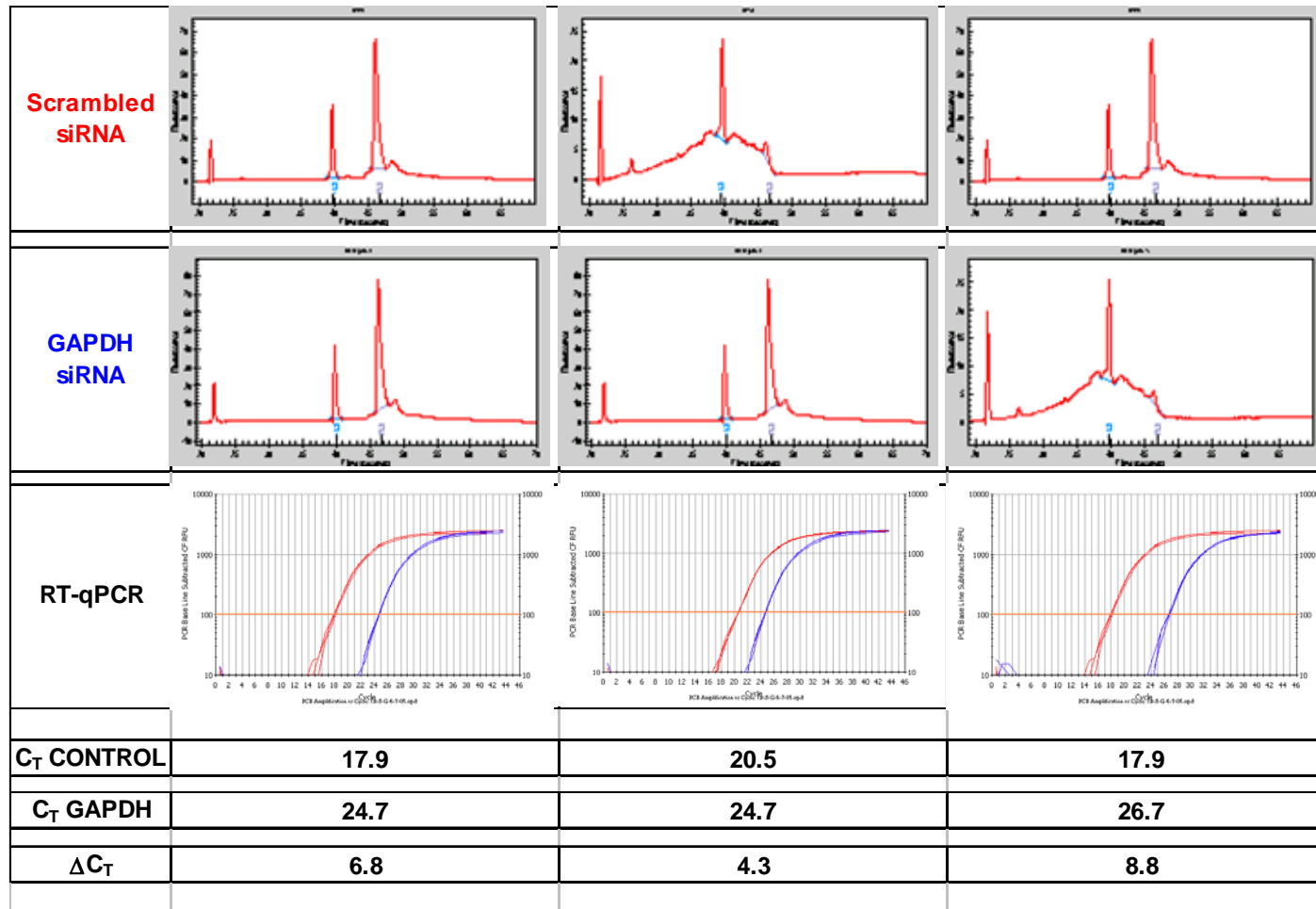
High



Recommended dosage

Check quality of RNA

The quality of the extracted RNA can have an impact on experimental results.

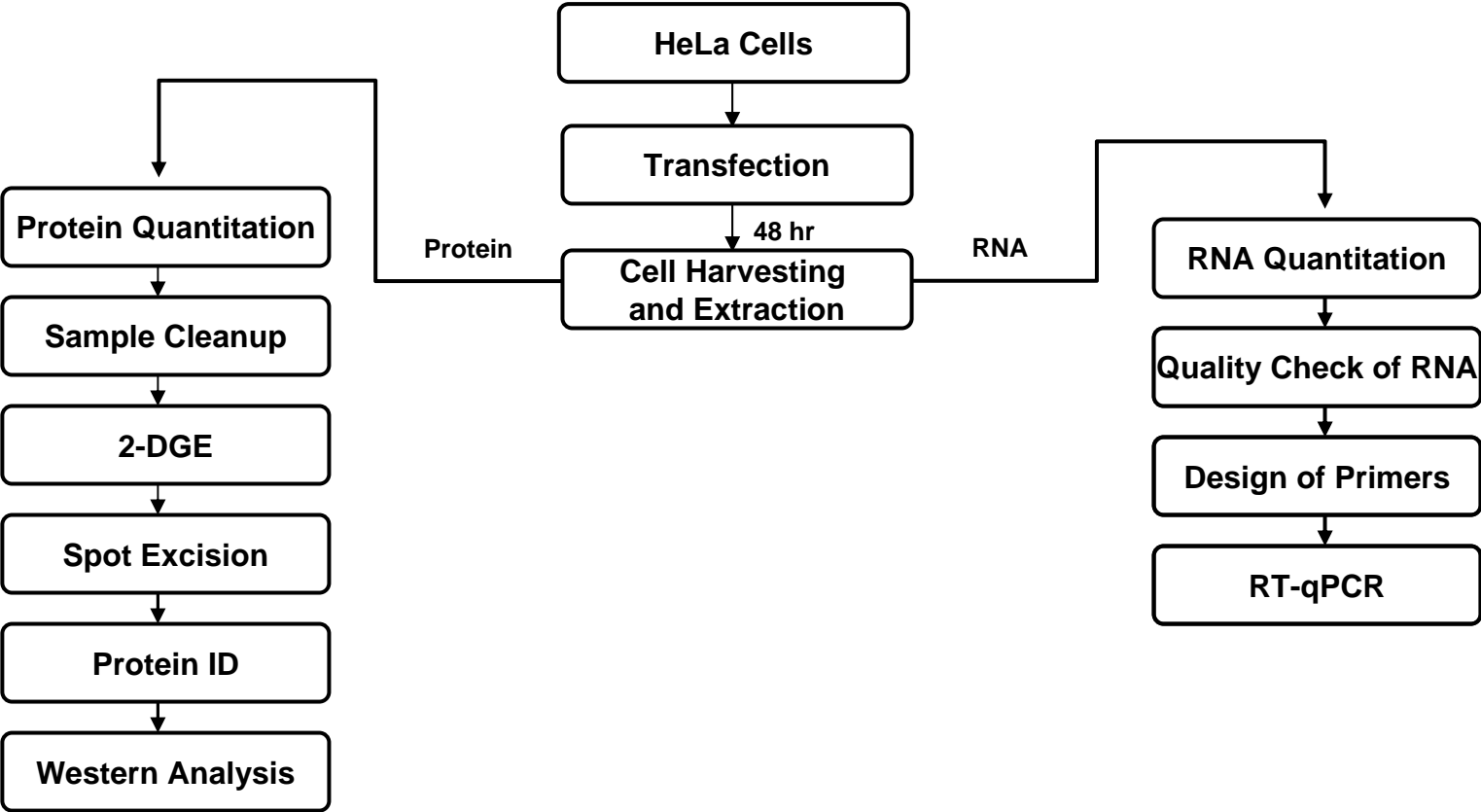


β -actin Gene Silencing via siRNA and its Effects on the Protein Profiles

Teresa Rubio, Ning Liu, Katrina Academia, Tim Wehr, Yuan Yan, Joseph Terefe, Todd Yeck, Eli Hefner
Keith Hamby, and Aran Paulus (Bio-Rad Laboratories)

Background:

- **Actin filaments – major cytoskeleton structures in cells**
- **Cell physiological behaviors – migration, proliferation, & differentiation**
- **Proper function depends on dynamic filament assembly and disassembly**
- **Studies have identified many proteins interacting with actin to regulate the cytoplasm by cross-linking, bundling, capping, or severing actin filaments.**

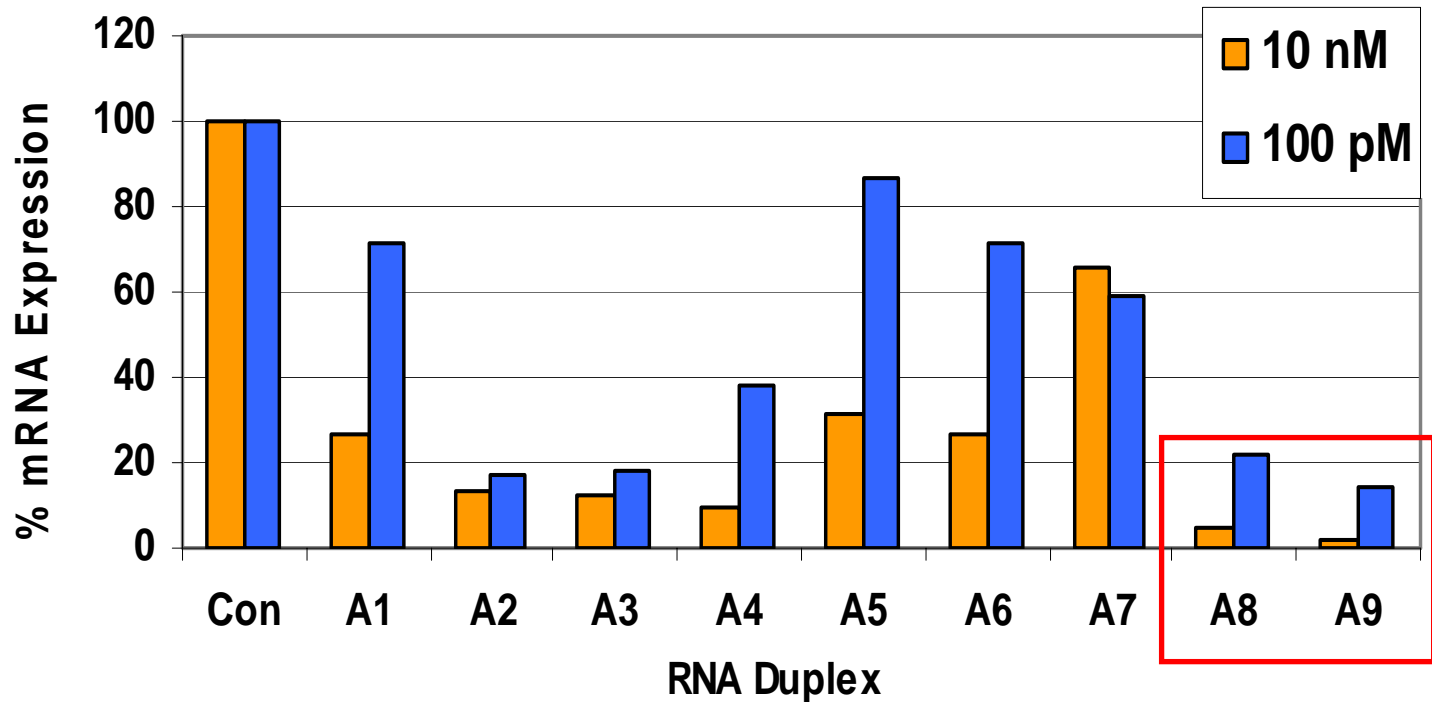


Collaborated with Integrated DNA Technologies (IDT)

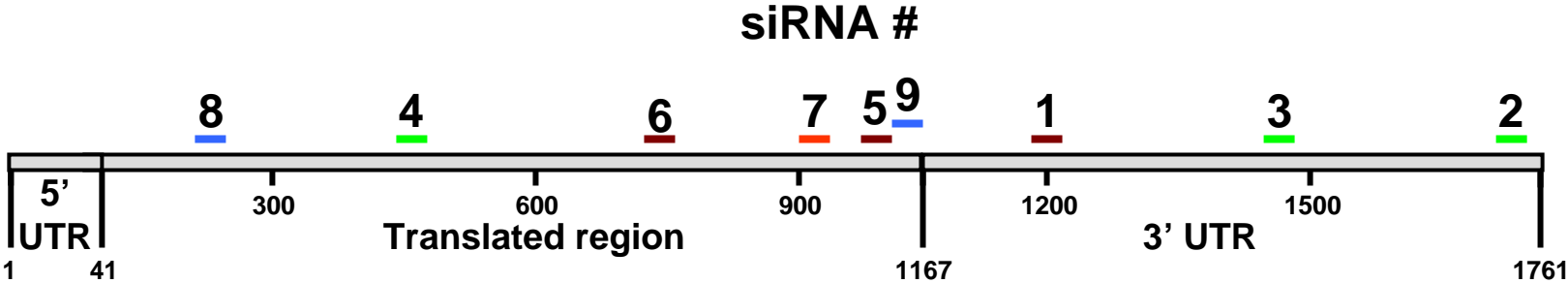
- IDT synthesized 9 Dicer Substrates (siLentMers)
9-sequences cover β -actin mRNA sequence
- Bio-Rad designed primer pairs and screened sequences for efficacy using RT-qPCR
Screen performed with 10nM and 100pM to assess potency

Goal: Select two effective sequences for use in the study (phenotypic confirmation)

- ✓ Using RT-qPCR, we tested nine anti-actin siLentMers
- ✓ Tested at 10nM and 100pM to assess efficacy



Distribution of siLentMers along the mRNA sequence



- <10%
- 10-20%
- 20-40%
- >40%

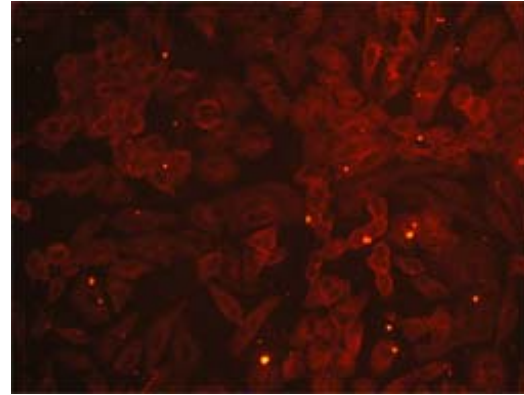
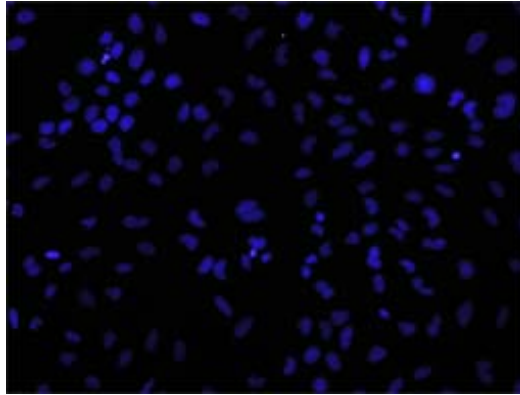
Remaining expression
after transfection with 10nM
Anti β -Actin siLentMer

Immuno-fluorescence

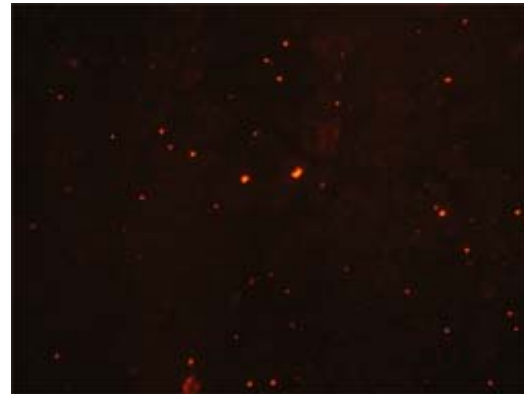
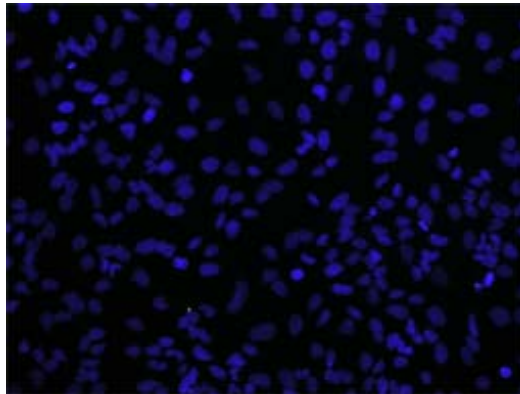
Hoechst dye

β -actin antibody

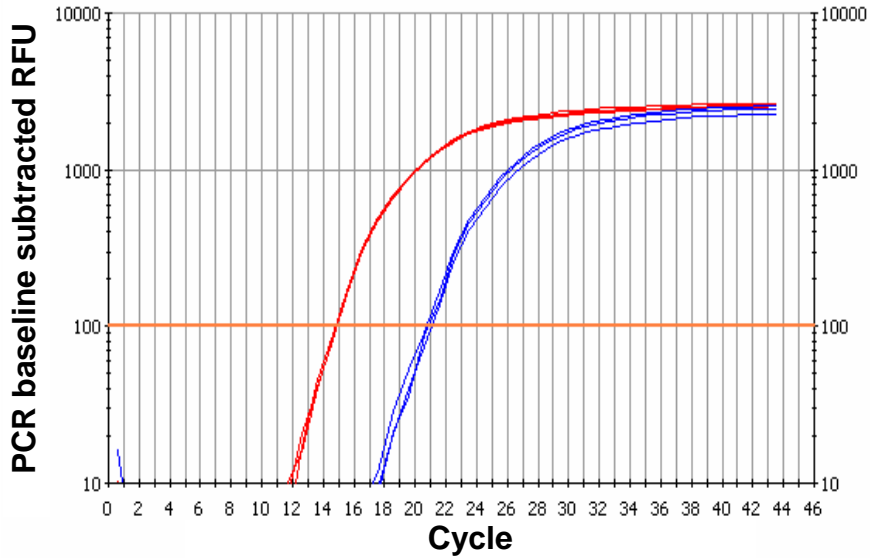
anti-eGFP
siLentMer



anti-actin
siLentMer



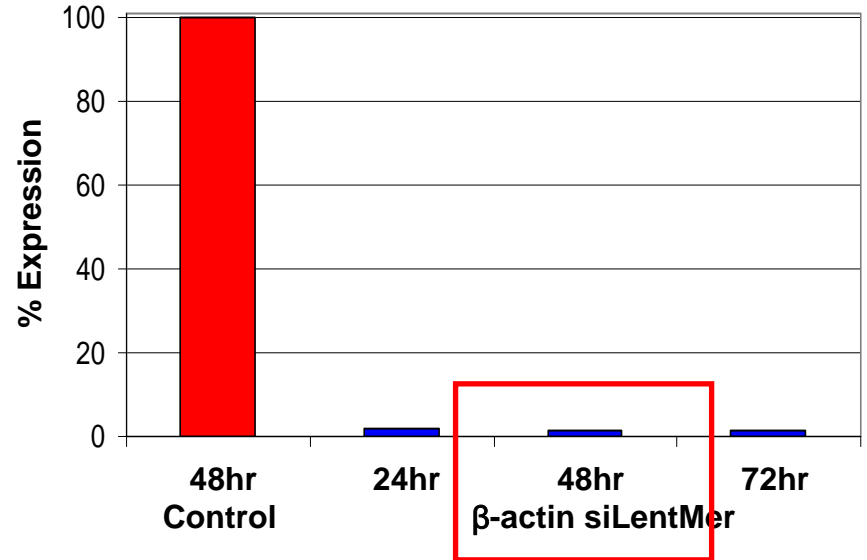
RT- qPCR Assay Validation



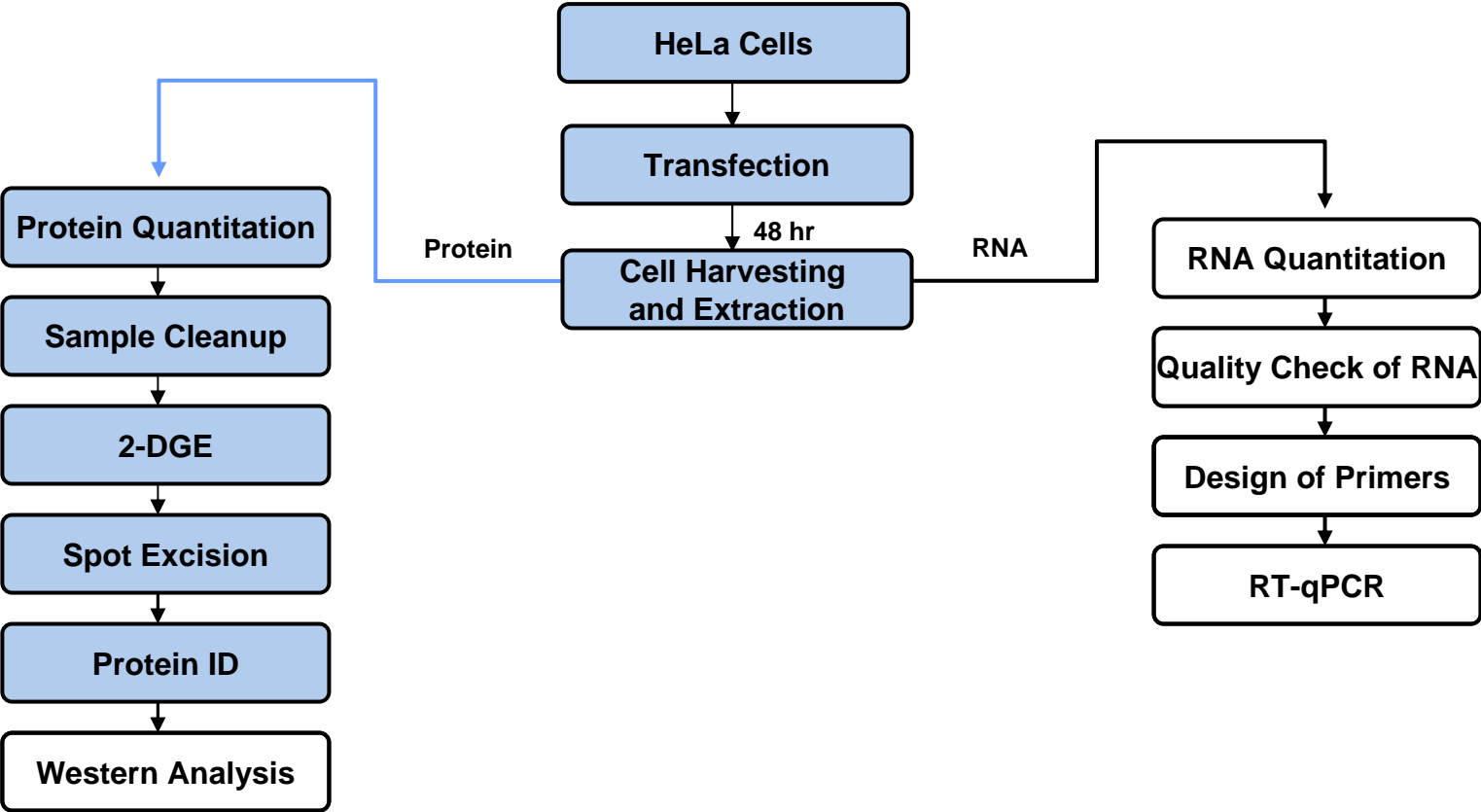
■ β -actin mRNA, $C_T = 21$

■ eGFP (control), $C_T = 14.9$

$$\Delta C_T = 6.1$$



> 95% Knockdown



Day 1: Seed 0.675×10^6 HeLa cells in 10ml of media on a 100mm plate

Day 2: Transfection.

Prepare siLentMer complexes with siLentFect and incubate 20 min.

Add complexes to culture.

Day 3: Change media to remove complexes, and add 10 ml of fresh media

Day 3 and 4: Extract protein

Remove media, wash cells with PBS.

Add 1ml of Laemmli buffer per plate.

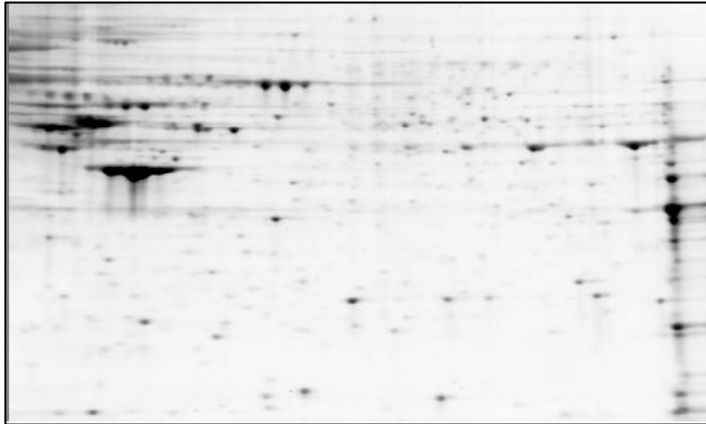
Collect the extract into eppendorf tubes.

Boil for 3min, and keep it at -20°C

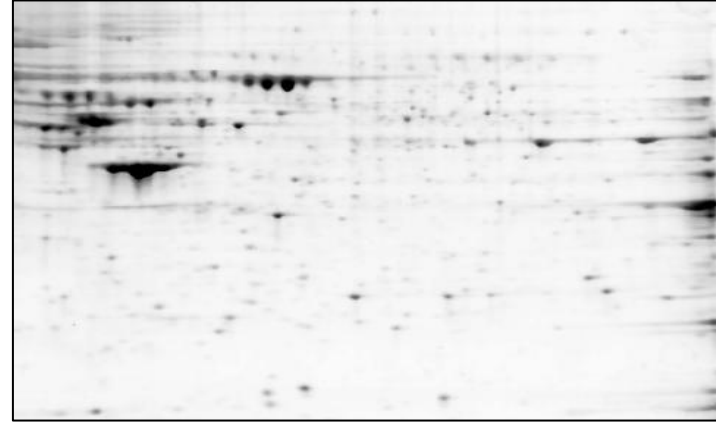
2-DGE Time Course Study

RNAi Solutions

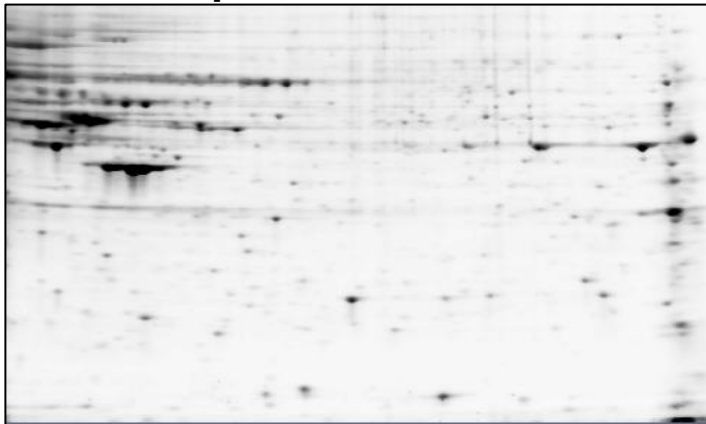
24hr. Control
Spot count: 343



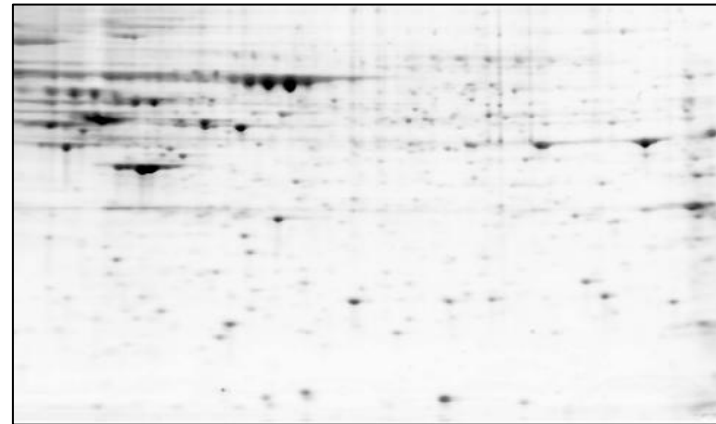
48hr. Control
Spot count: 344



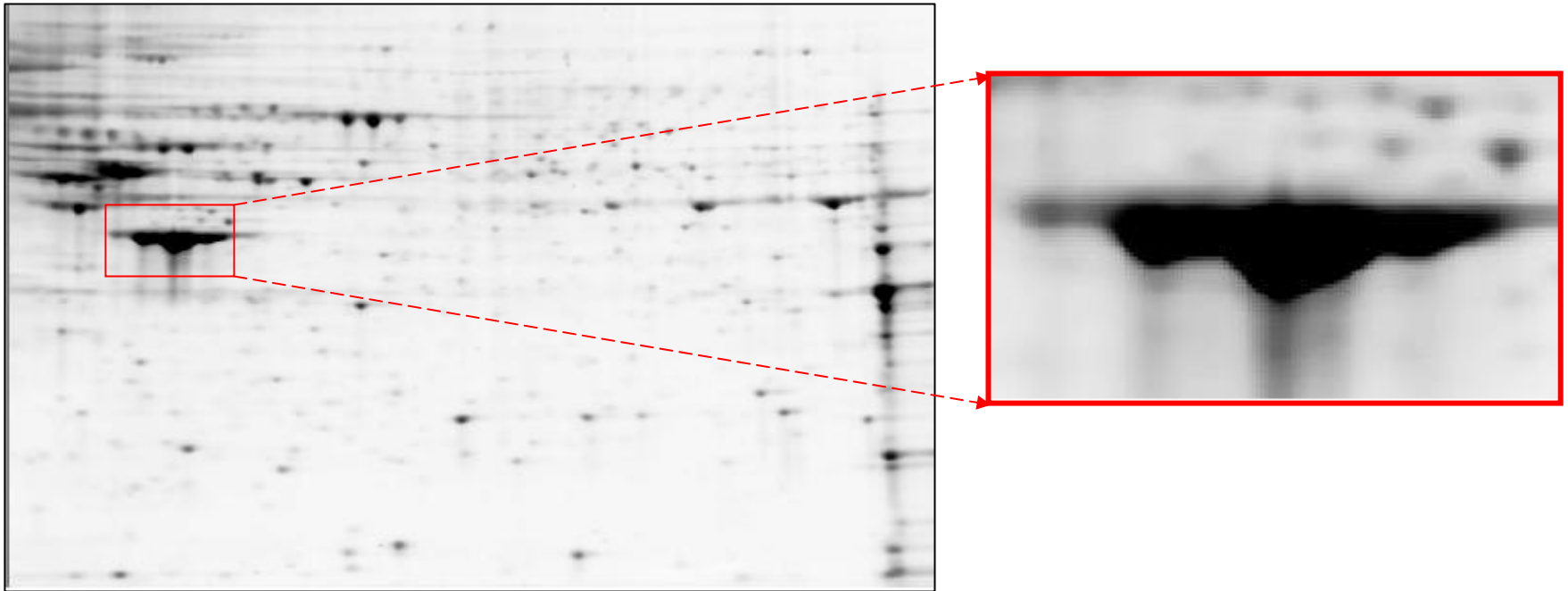
24hr. (Test)
Spot count: 348



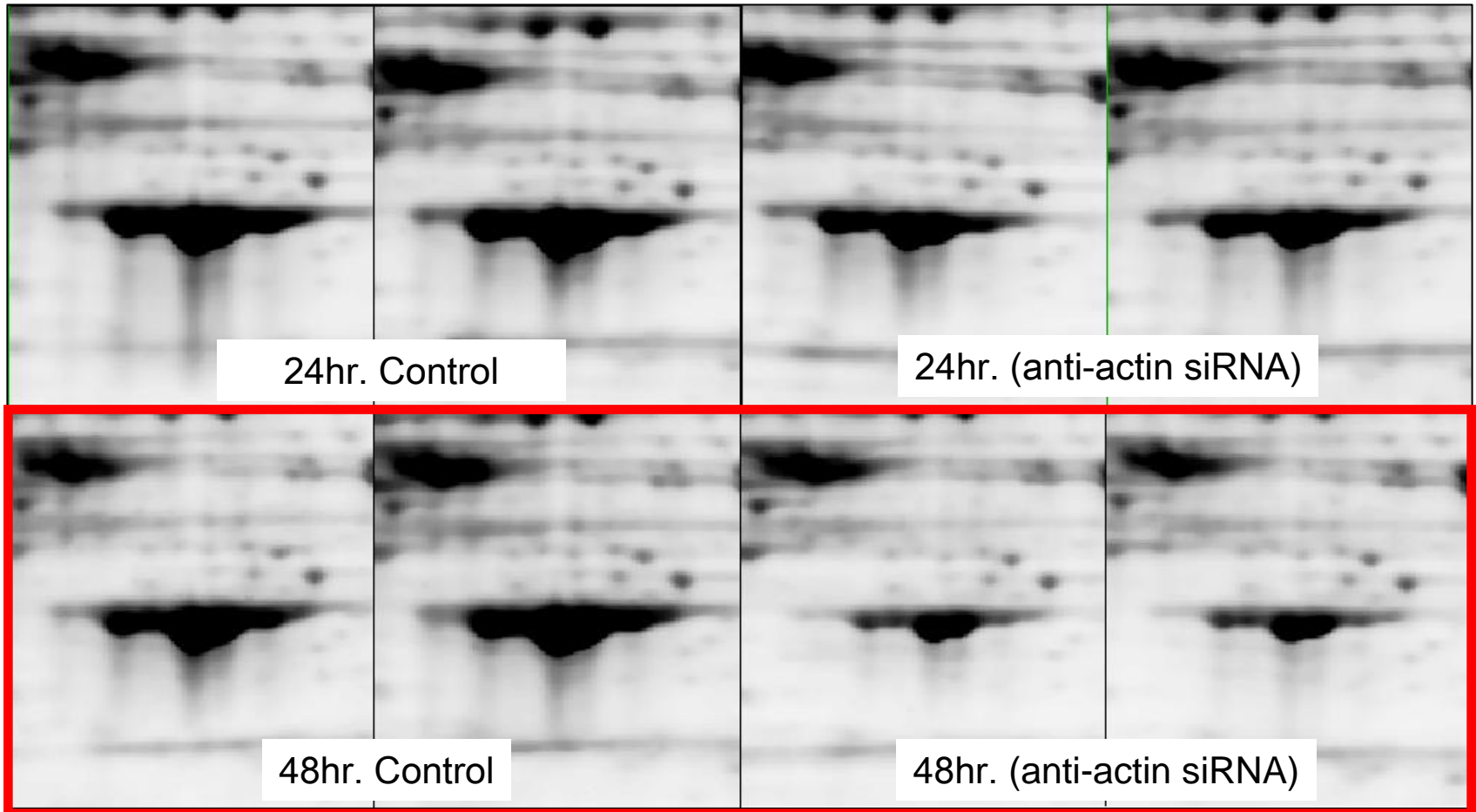
48hr. (Test)
Spot count: 340



24hr. Control



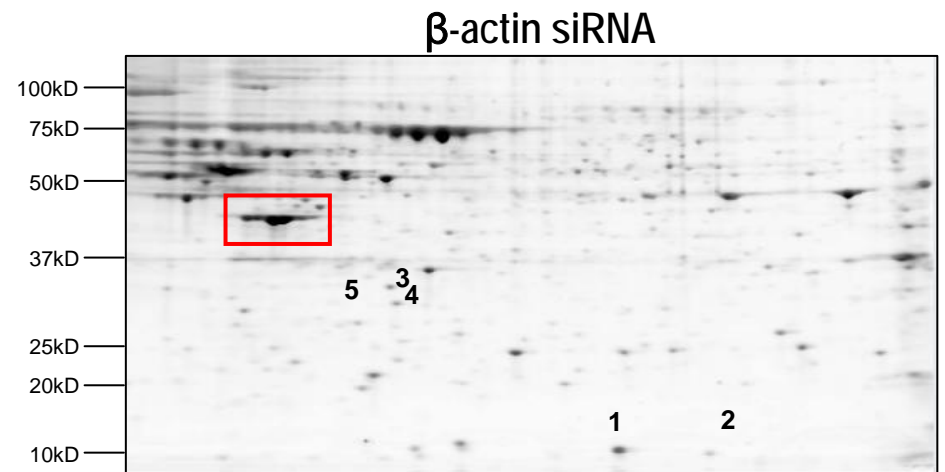
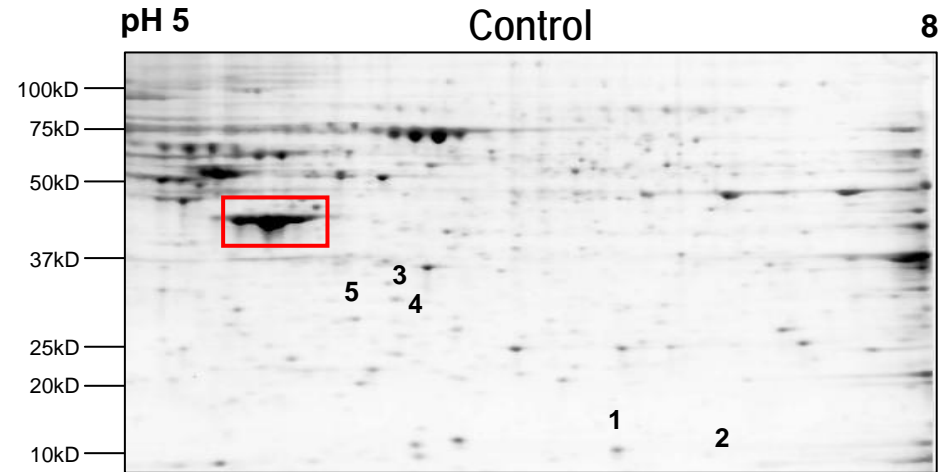
β -actin, pI 5.5, MW 41.7kDa



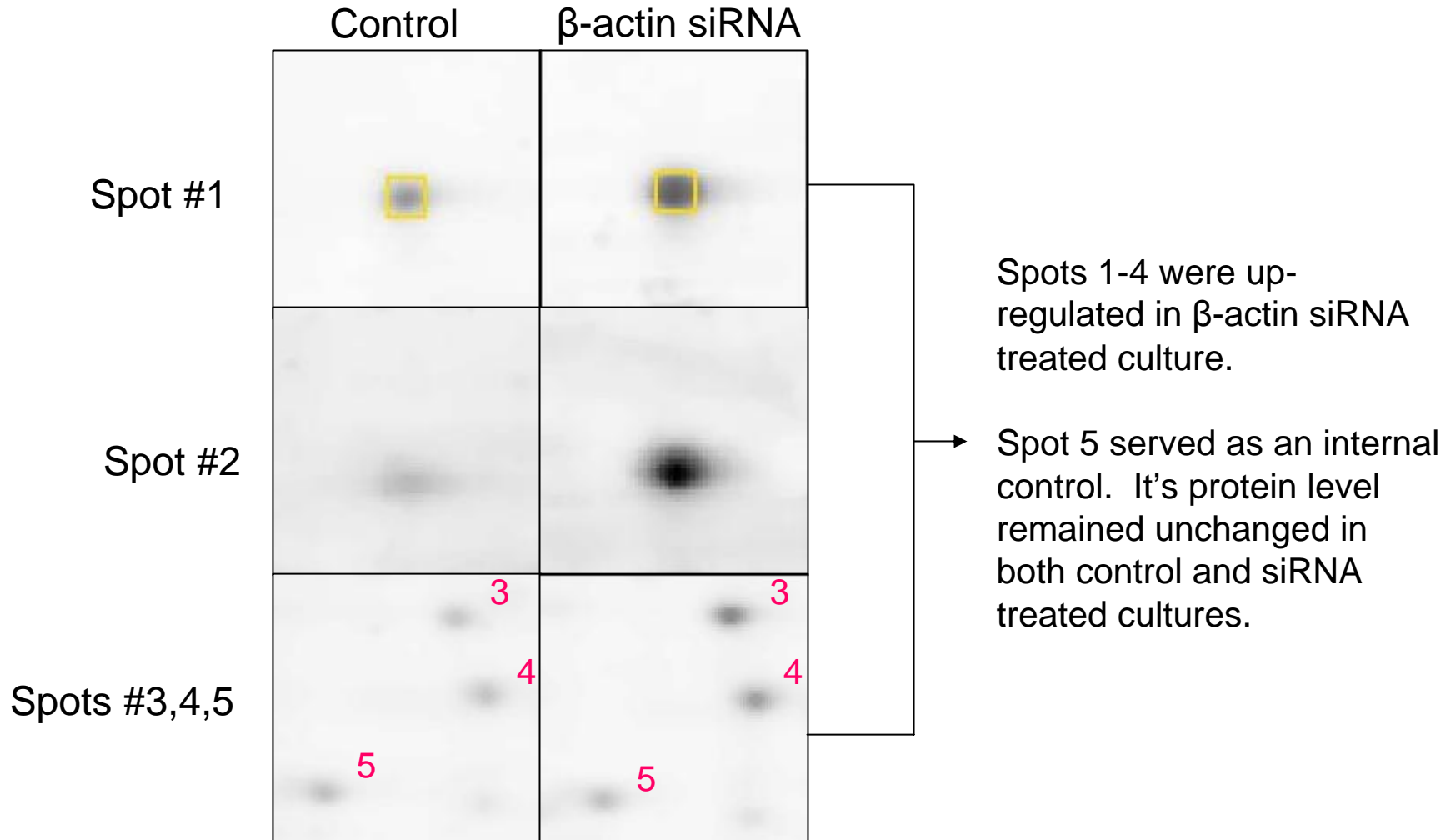
2-DGE Protocol

- 80ug of protein load (in duplicate)
- 11cm, pH 5-8 IPG strips
- 8-16% Tris-HCl Criterion gels
- Flamingo Pink stain
- PDQuest analysis

Four spots identified through 2DGE gel analysis relative to internal control (5).



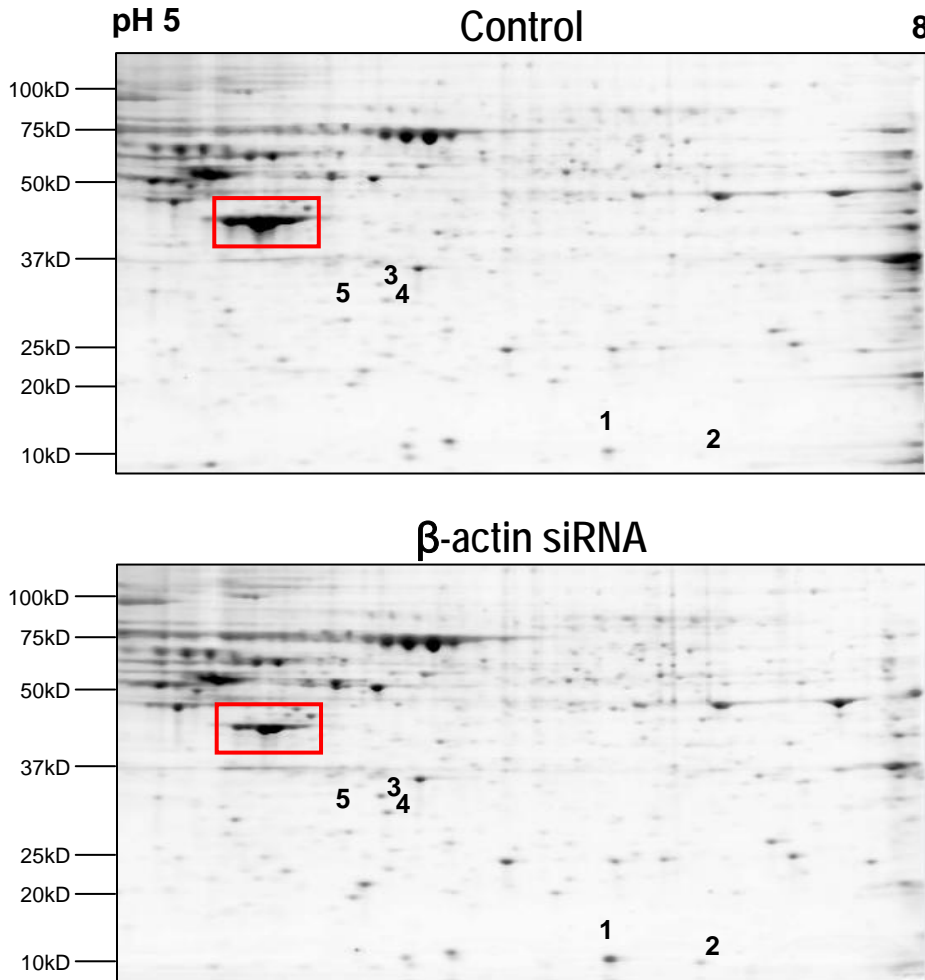
2-DGE Analysis



Sample	P ¹	US ²	% Coverage	Database Mass (kDa)	Database pI	Identity
Control	1.9 e-013	114.3	32.6	41786.7	4.25	β -actin
siRNA-treated	2.7 e-012	102.2	26.4	41786.7	4.25	β -actin
Spot 1	2.2 e-014	70.4	36.1	18492	8.33	Cofilin
Spot 2	2.2 e-008	58.2	36.4	18494	8.33	Destrin
Spot 3	1.6 e-012	66.3	25.5	36354	6.00	Annexin A3
Spot 4	1.2 e-009	40.3	31.1	21094	9.20	CAPZB protein
Spot 5 <i>Internal control</i>	4.5 e-010	38.3	18.8	29787	4.25	Prohibitin

- Reversed-phase nanospray LC-MS-MS using an LTQ linear ion trap mass spectrometer
- Protein search in the human database using TurboSEQUENT with BioWorks 3.2 software

2- DGE Spot Summary



Spot 1: Cofilin
~2-fold increase

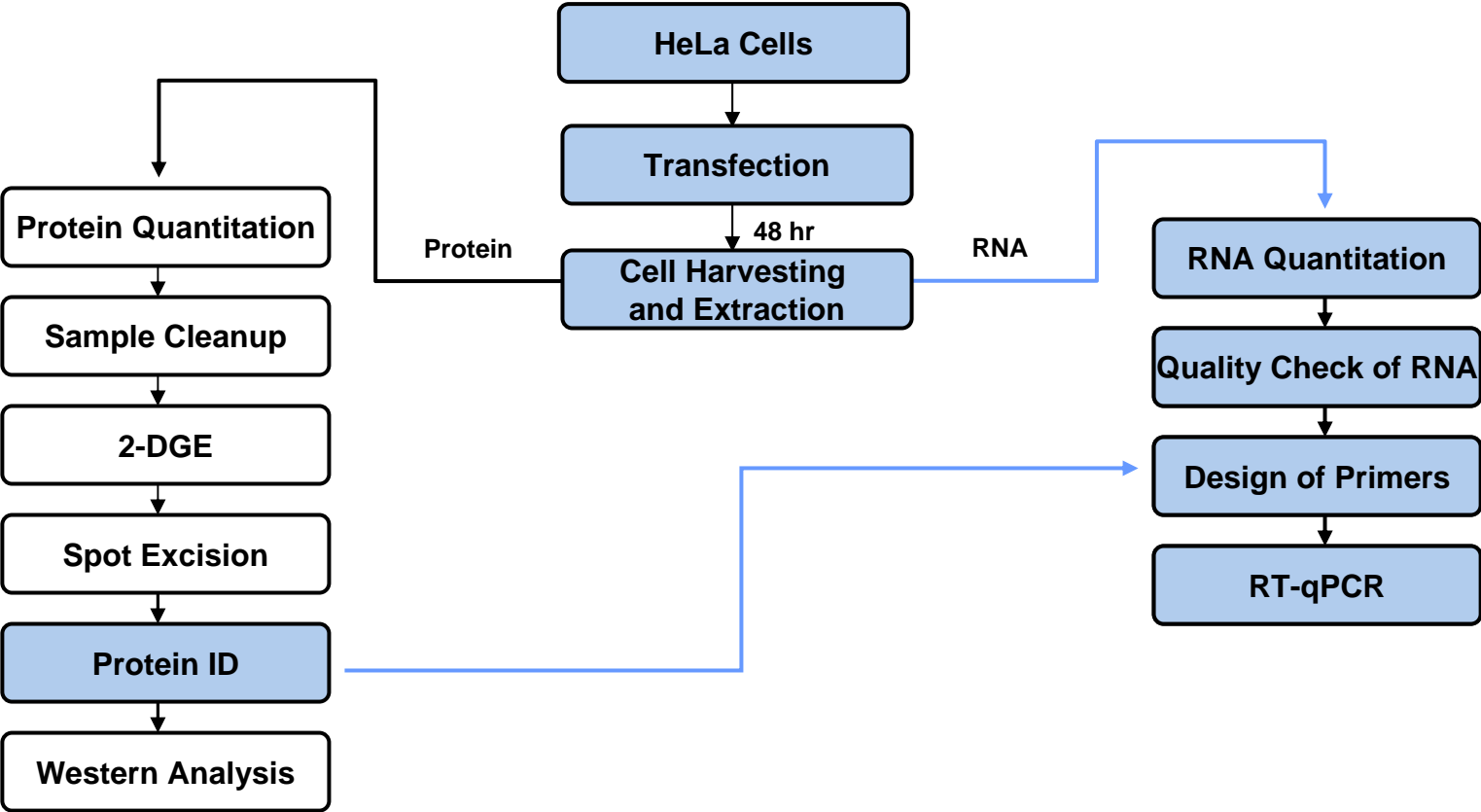
Spot 2: Destrin
~3-5 fold increase

Spot 3: Annexin A3
~2-fold increase

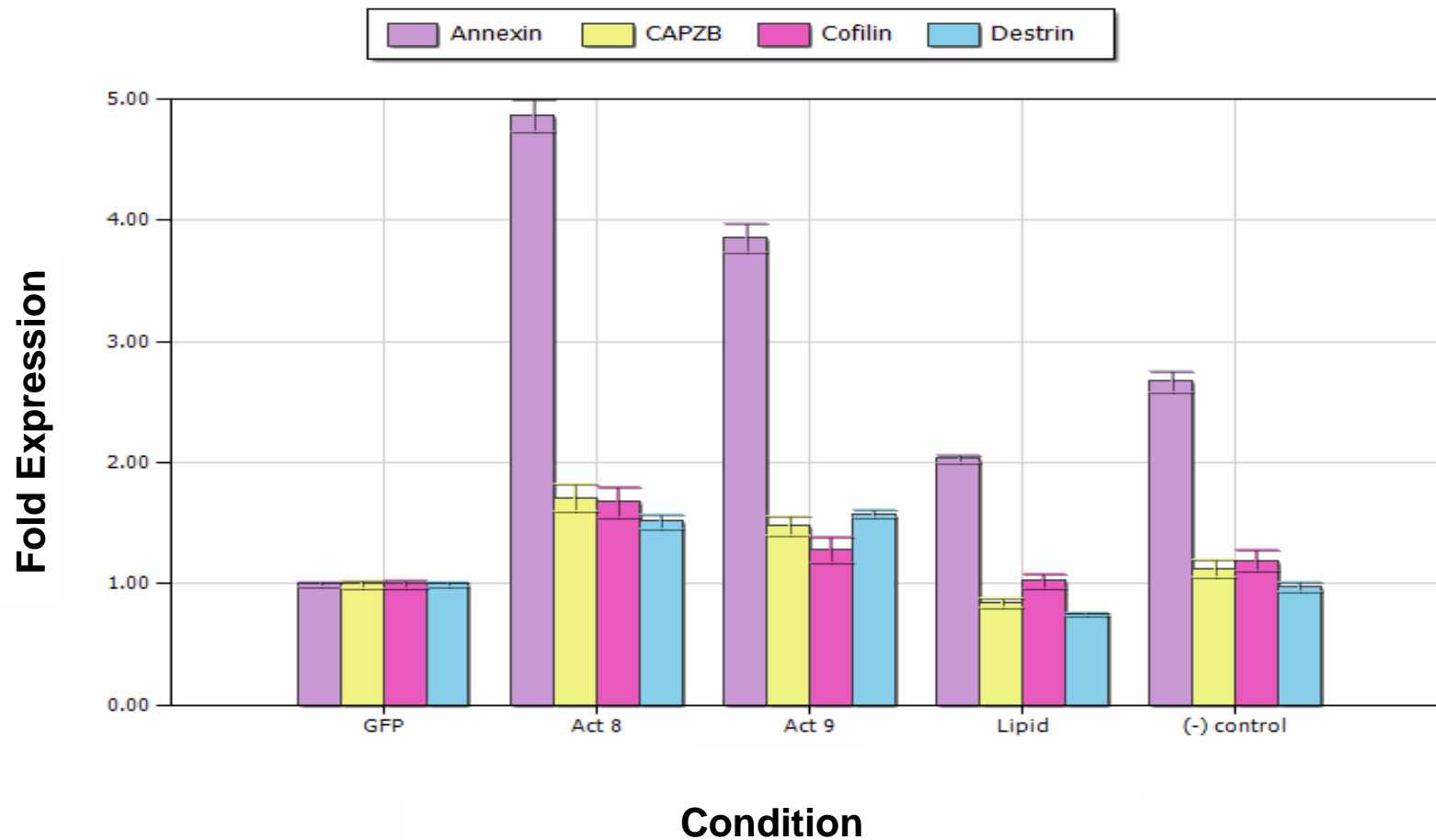
Spot 4: CAPZB
~2-fold increase

Spot 5: Prohibitin
no change

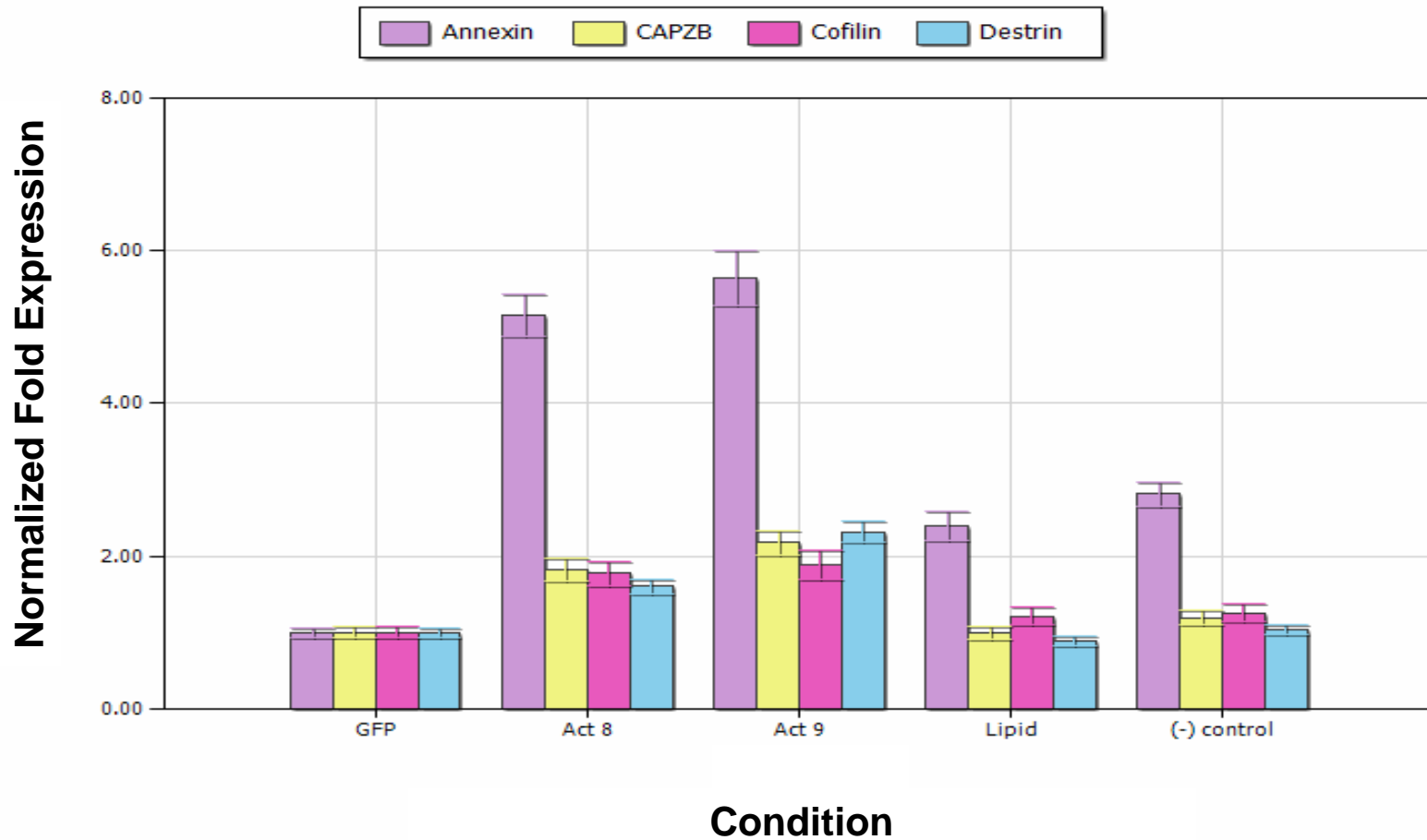
- Cofilin, an actin-binding protein, is an actin depolymerization factor; it binds to actin filaments and induces cleavage of the filaments.² It plays important role in remodeling the highly dynamic structure of actin filaments.³
- Destrin is an actin depolymerization factor; it has a function similar to that of cofilin.^{3,4}
- CAPZB is a subunit of the Cap Z protein. “Cap Z is a widely distributed, highly conserved, heterodimeric protein that binds to barbed end of actin filaments but does not sever filaments”.⁶
- Annexin A3, also known as lipocortin 3, is a calcium- and membrane binding protein located in early endosomes with unclear functions.⁵
- Prohibitin is localized to the mitochondria and may have a role in the maintenance of mitochondria function and protection against senescence.⁷



Normalized to input RNA

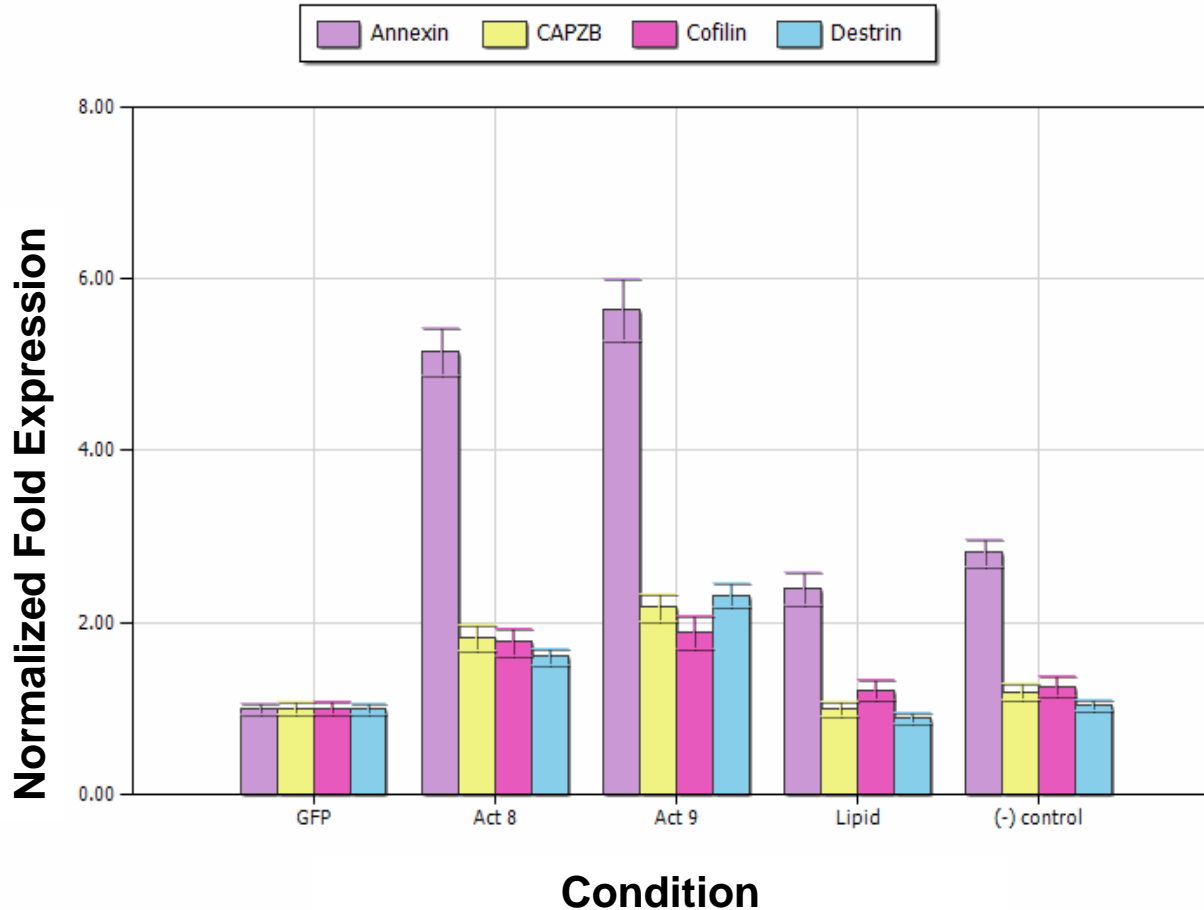


Normalized to geometric average of reference genes



Multiplex RT-qPCR Results

RNAi Solutions



Cofilin
~1.5 fold increase

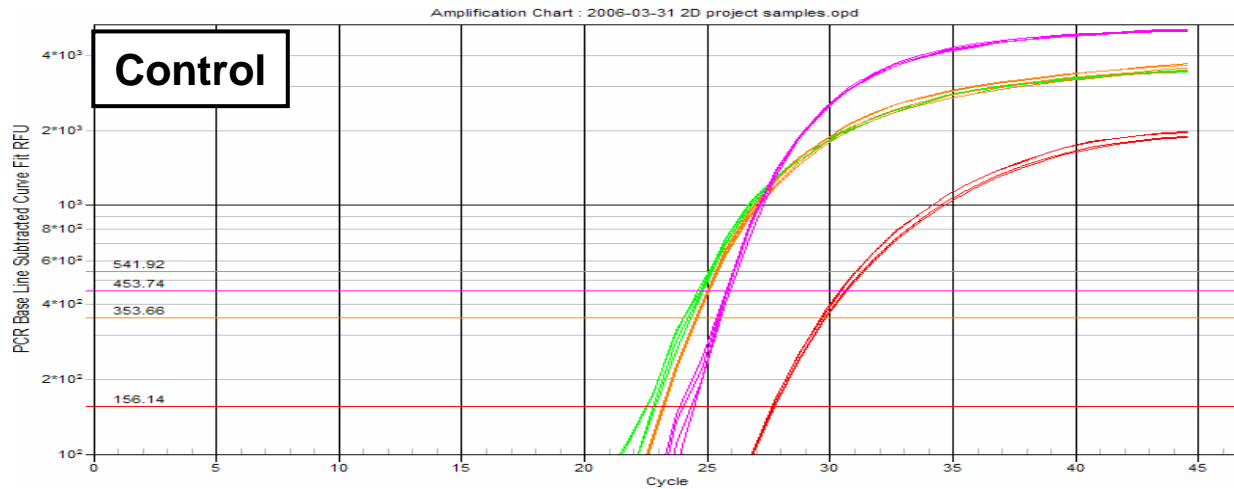
Destrin
~2 fold increase

Annexin A3
~5 fold increase

CAPZB
~2 fold increase

Validate 'Hits' with qPCR

Multiplex



Cofilin (FAM)

green

Destrin (CalOrange560)

orange

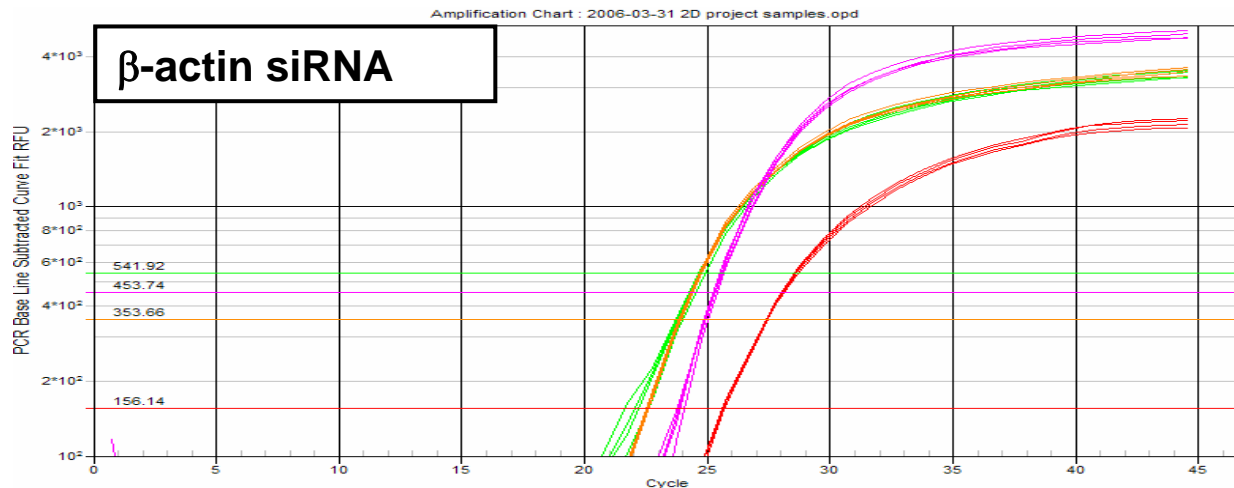
Annexin (CalRed610)

pink

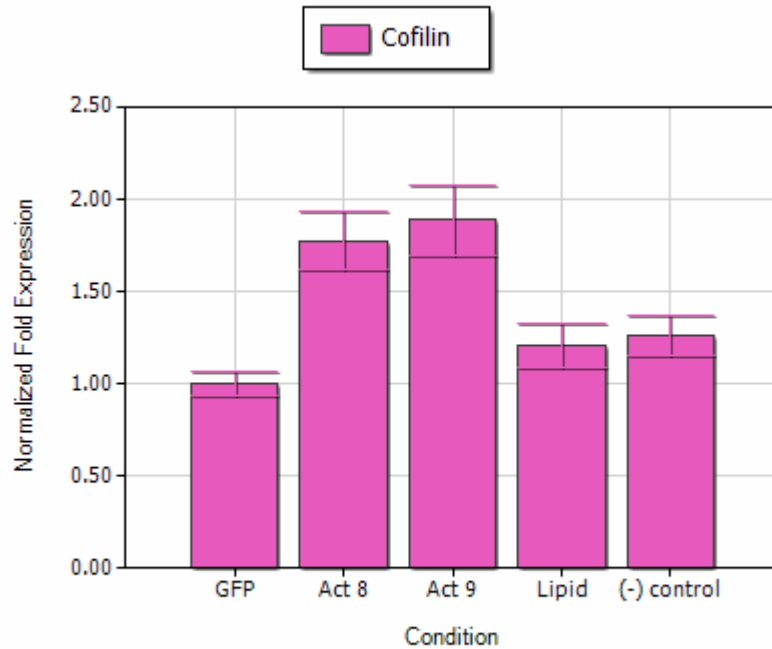
CAPZB (Quasar670)

red

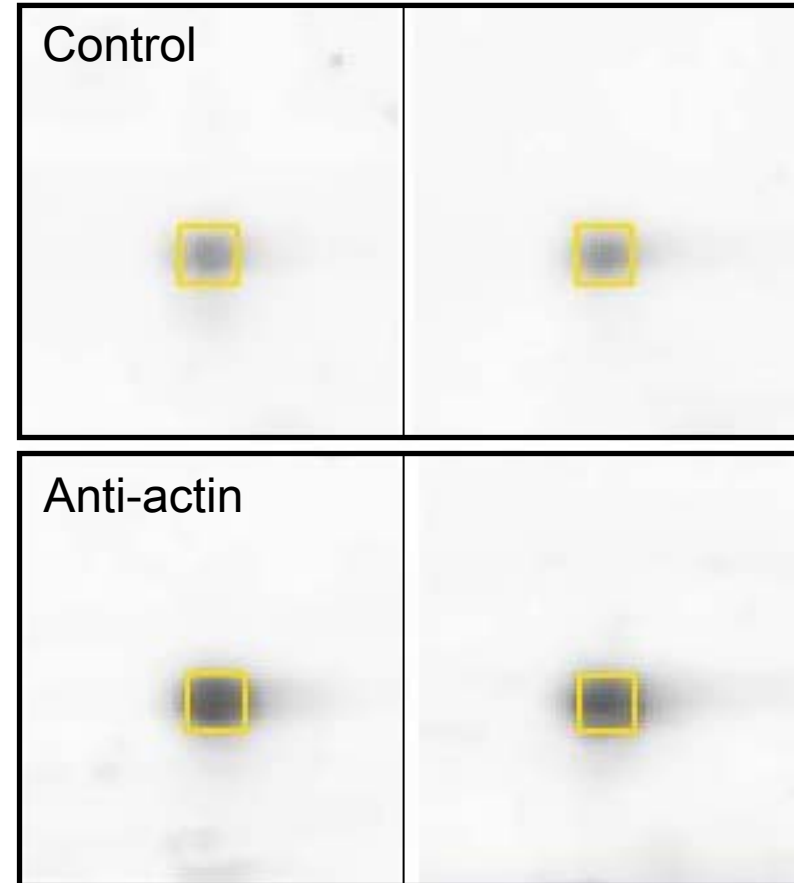
Normalization: GAPDH
Tubulin and 18S RNA



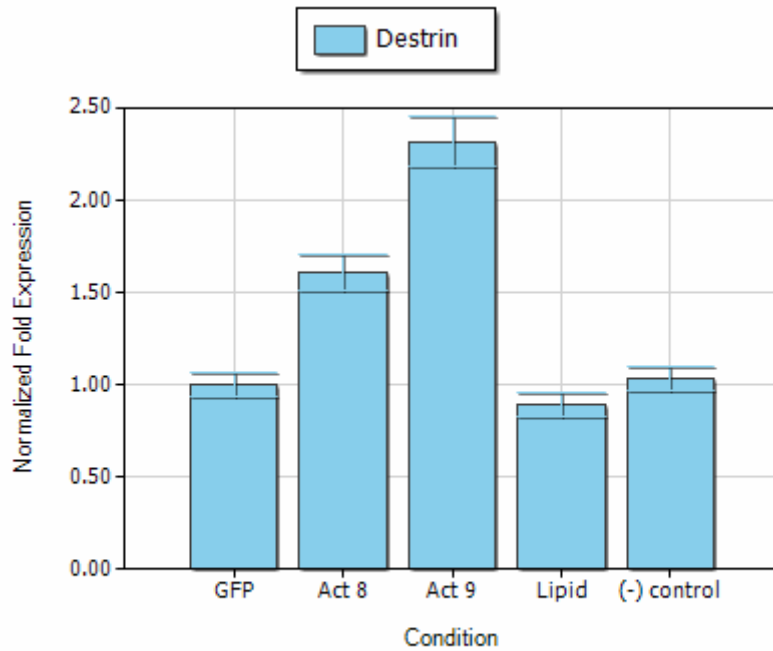
Spot #1: Close-up



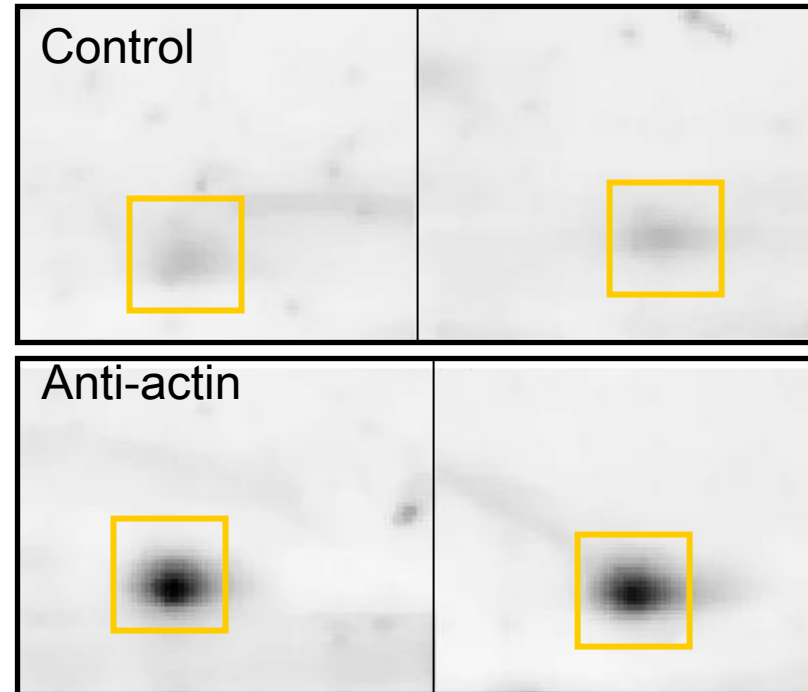
Gene Expression : combined Multiplex data.gxd



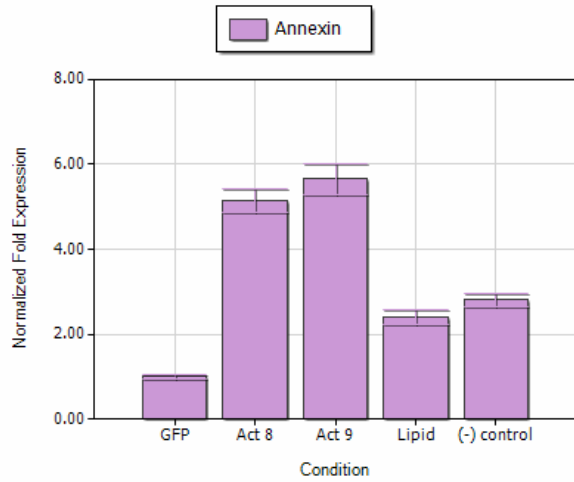
Spot #2: Close-up



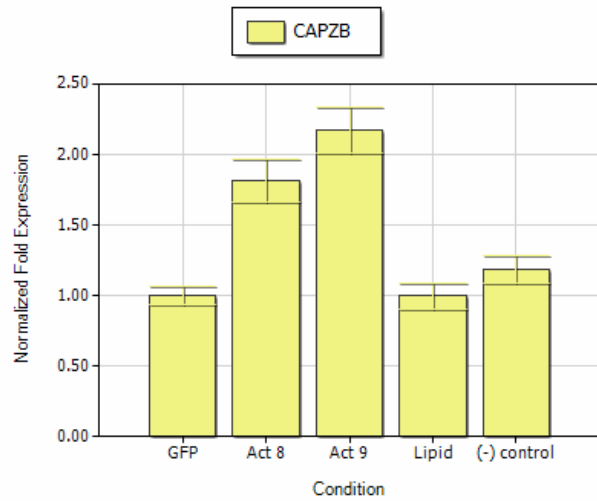
Gene Expression : combined Multiplex data.gxd



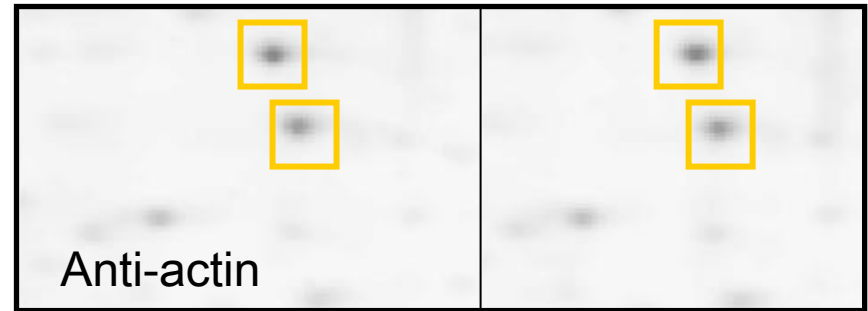
Spot # 3 & 4: Close-up



Gene Expression : combined Multiplex data.gxd



Gene Expression : combined Multiplex data.gxd



Target

2-DGE Analysis

RT-qPCR Analysis

Cofilin

2 Fold ↑

1.5 Fold ↑

Destrin

3 – 5 Fold ↑

2 Fold ↑

Annexin A3

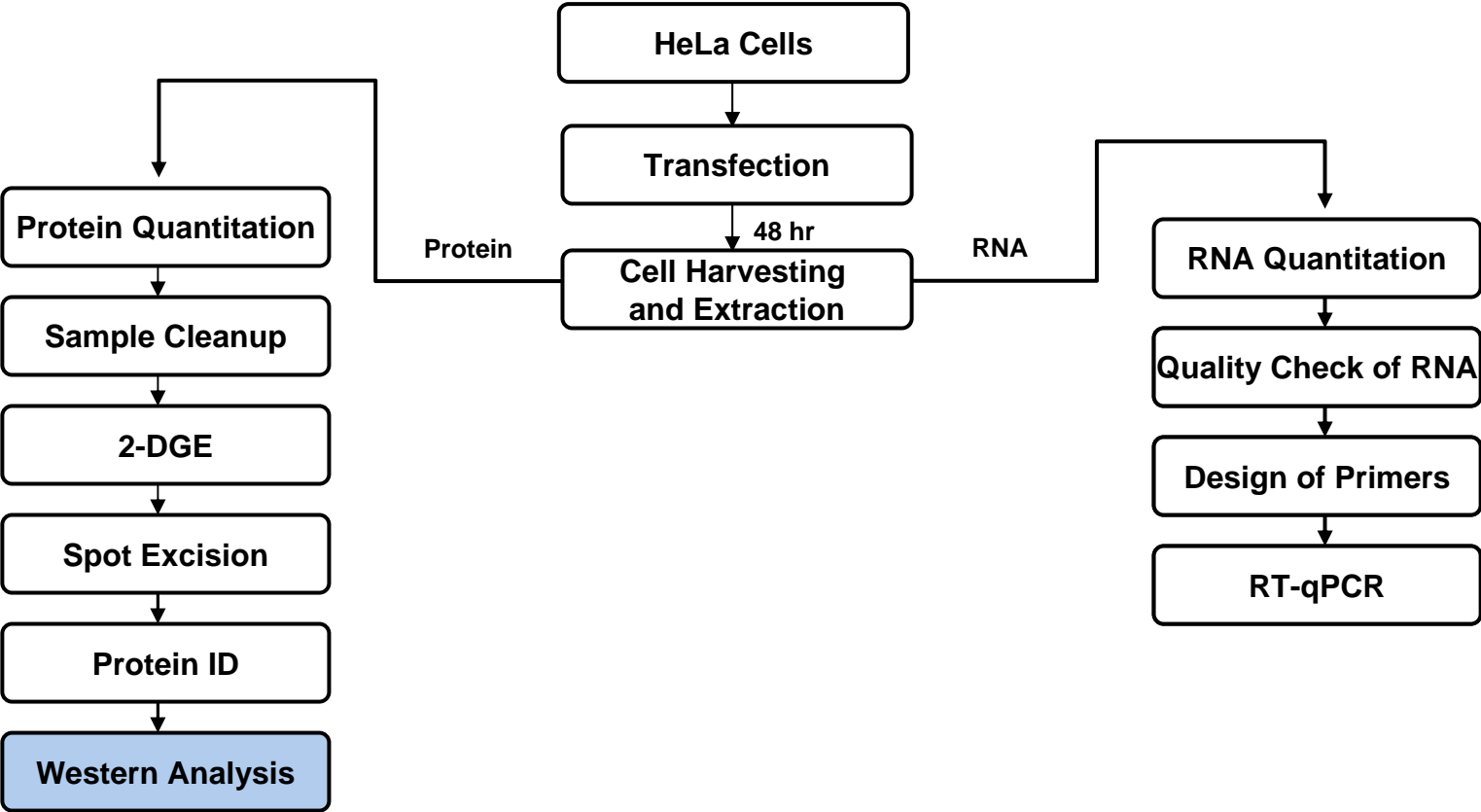
2 Fold ↑

5 Fold ↑

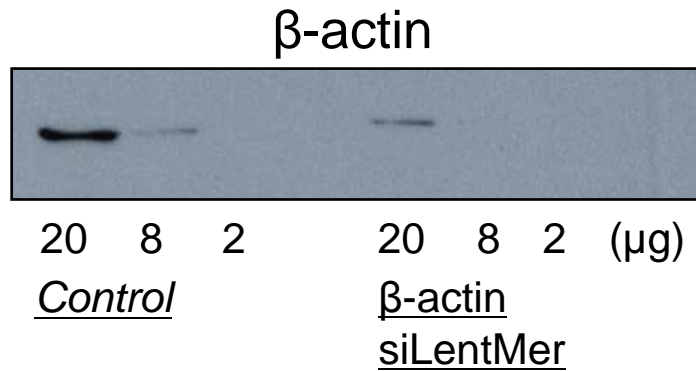
CAPZB

2 Fold ↑

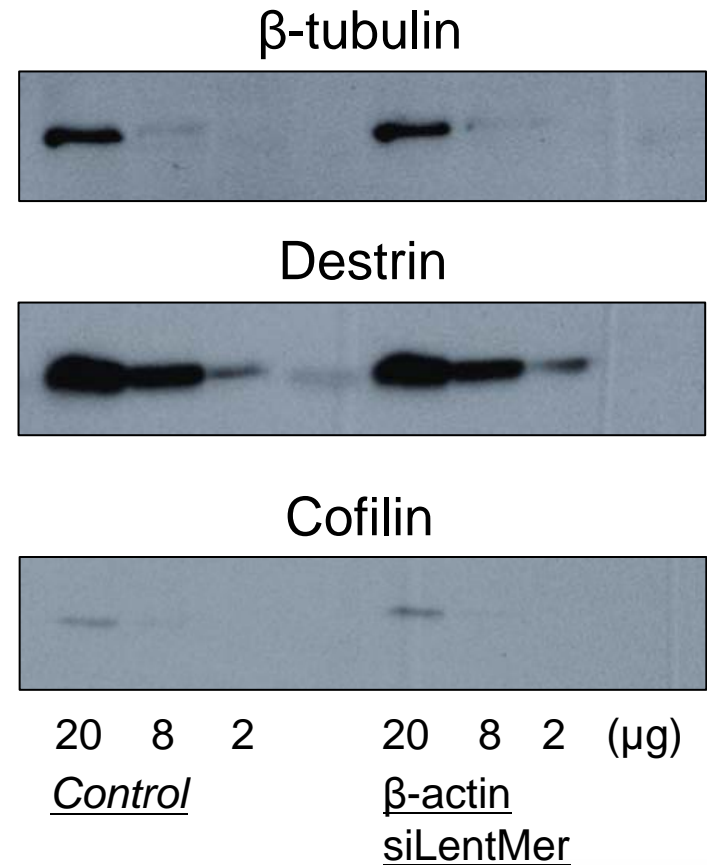
2 Fold ↑



Down-Regulated Expression



No Change in Expression



Down-Regulated Expression

β -actin



20 8 2 20 8 2 (μ g)

Control

β -actin

siLentMer

Phosphorylation

Phosphorylated Cofilin



20 8 2 20 8 2 (μ g)

Control

β -actin

siLentMer

No Change in Expression

β -tubulin



Destrin



Cofilin



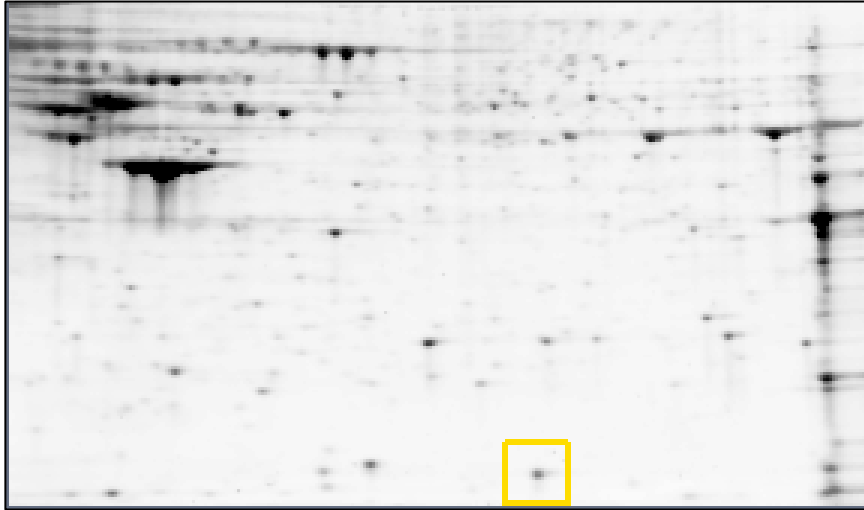
20 8 2 20 8 2 (μ g)

Control

β -actin

siLentMer

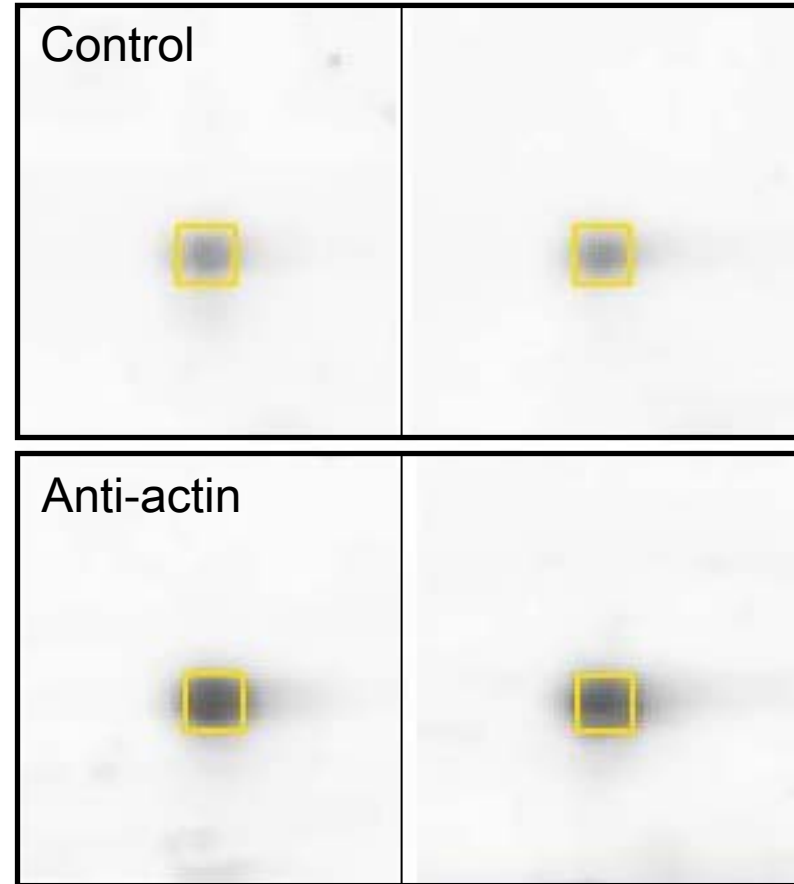
Spot #1: Close-up



Spot #1 Profile

pI: ~8

Mass: ~18,000 kDa



Knocking down β -actin expression in Hela cells:

- Induced protein level changes in several proteins that directly interact with the actin filaments.
 - *Cofilin* (actin depolymerization factor)
 - *Destrin* (actin depolymerization factor)
 - *CAPZB* (actin filament capping protein)
- Cofilin protein is inactivated through phosphorylation rather than transcription down-regulation.
- This process demonstrates the benefit of using three types of analysis methods to interpret gene knockdown.

Knocking down β -actin expression in Hela cells:

- Like cofilin, is destrin phosphorylated when actin is silenced?
- Is Annexin A3 transcript increase due to lipid transfection or does it interact with actin?
- Why is cofilin phosphorylated and not down-regulated at the expression level when its target protein, actin, is suppressed?
- How do kinases LIMK and TESK and phosphatase Slingshot respond to actin silencing?

Team Members:

 **Bio-Rad Laboratories, Inc.** - Teresa Rubio, Joseph Terefe, Eli Hefner, Michael Sturges, Steve Kulisch

 **Integrated DNA Technologies, Inc. (IDT)** - Mark Behlke, Scott Rose, Andy Peek, John Havens