Quantitative immunofluorescence. FFPE whole-tissue sections, tissue microarrays (TMAs) and cell pellets were processed as follows; briefly, sections were baked for 30 minutes at 60° C and underwent two 20-minute washes in xylenes. Slides were rehydrated in two 1-minute washes in 100% ethanol followed by one wash in 70% ethanol and finally rinsed in streaming tap water for 5 minutes. Antigen retrieval was performed in sodium citrate buffer pH.6, for 20 minutes at 97°C in a PT module (LabVision). Endogenous peroxidases were blocked by 30-minute incubation in 2.5% hydrogen peroxide in methanol. Nonspecific antigens were blocked by a 30-minute incubation in 0.3% BSA in TBST. Slides were then incubated with the target primary antibody (ZNF71 Abcam; ab87250), as well as pan cytokeratin (AE1/AE3) overnight at 4C diluted at 1:100 to define the tumor compartment.

Primary antibodies were followed by incubation with Alexa 546–conjugated goat anti-mouse secondary antibody (Life Technologies) diluted 1:100 in rabbit EnVision reagent (Dako) for 1 hour. ZNF71 signal was amplified with Cy5-Tyramide (Perkin Elmer) for 10 minutes, and then nuclei were stained with 0.05 mg DAPI in BSA-tween for 10 minutes. Slides were mounted with ProlongGold (Life Technologies). Two TBS-T and one TBS wash was performed between each step after the primary antibody.

Immunofluorescence was quantified using automated quantitative analysis (AQUA) Fluorescent images of DAPI, Cy3 (Alexa 546-cytokeratin), and Cy5 (ZNF71) for each TMA spot were collected. Image analysis was carried out using the AQUAnalysis software (Navigate Biopharma Inc.), which generated an AQUA score for each compartment by dividing the sum of target pixel intensities by the area of the compartment in which the target is measured. AQUA scores were normalized to the exposure time and bit depth at which the images were captured, allowing scores collected at different exposure times to be directly comparable. Specimens with less than 5% tumor area per region of interest were not included in AQUA analysis for not being representative of the corresponding tumor specimen.

Quantification of ZNF71 on NSCLC cohort YTMA 250

Yale University, School of Medicine

STS Lab

November 11, 2015

Objectives and methods

- Testing and titration of ZNF71 on NSCLC test TMAs as well as Western blot for ZNF71 was performed and reported previously.

- The Yale NSCLC cohort YTMA 250-3-5 was stained and quantified for ZNF71 expression levels using previously optimized and standardized conditions.

- Quantification of staining is performed using the AQUA method of quantitative immunofluorescence (QIF)

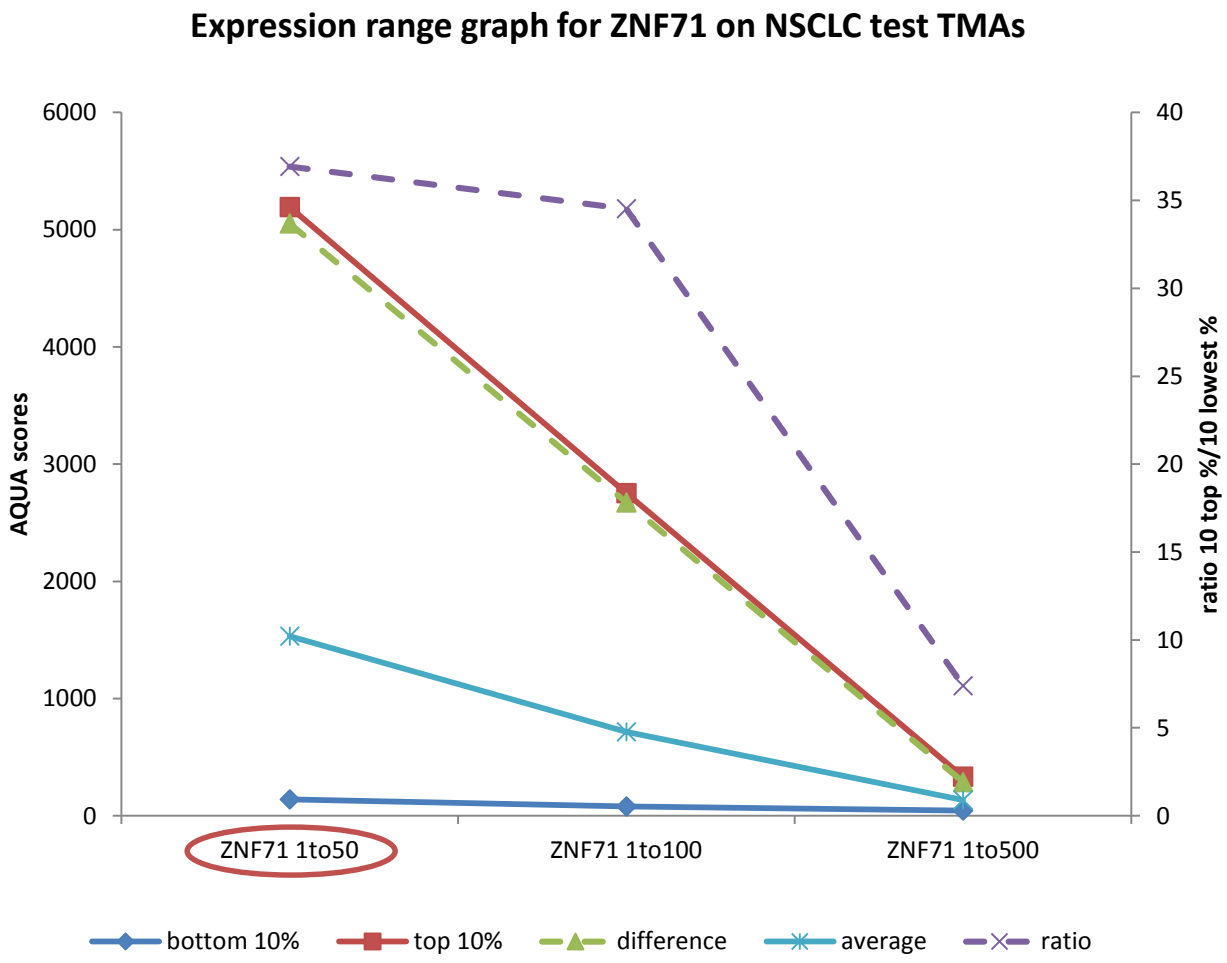
- Antibody: **Anti-ZNF71 antibody ab87250;** abcam rabbit polyclonal antibody; antigen retrieval Citrate buffer pH6; incubation over night at 4 degree Celsius at a titer of 1 to 50; protein blocking performed before antibody incubation with 2% BSA for 1 hour at room temperature.

Cohort Characteristics of YTMA 250:
Block YTMA 250-3-5

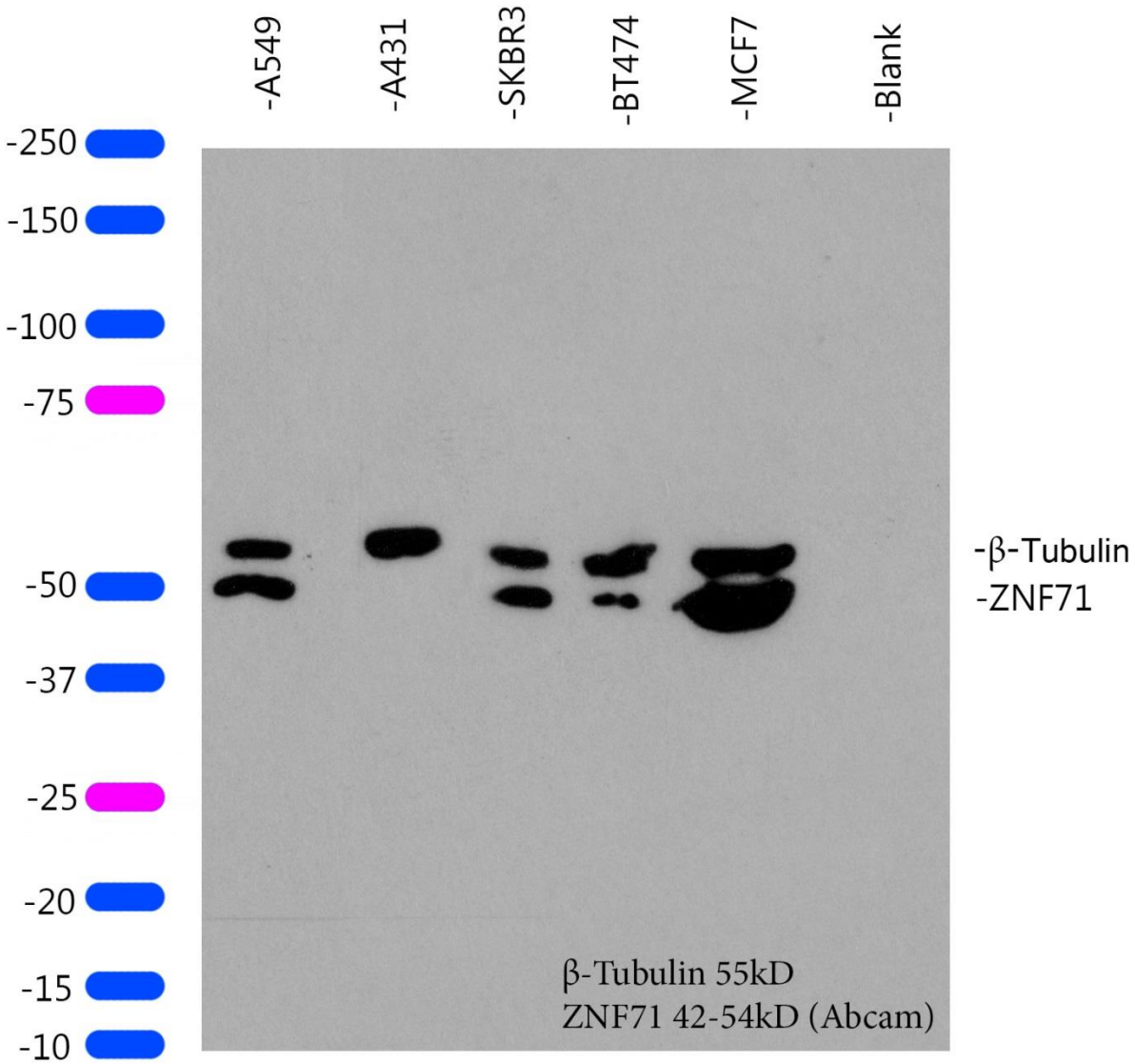
Total spots on the array: 314
Patient samples: 298, 8 patient samples represented in 2 fold redundancy, 8 controls

Parameter	YTMA250
All Patients (%)	298 (100%)
Date of diagnosis	2004-2011
Age (Years)	
< 70	179 (60%)
≥ 70	119 (40%)
Gender	
Male	128 (42.9%)
Female	170 (57.1%)
Smoking Status	
Never	47 (15.8%)
Smoker	234 (78.5%)
Unknown	17 (5.7%)
Histology	
Adenocarcinoma	180 (60.4%)
Squamous	74 (24.8 %)
Other	44 (14.8%)
Primary Tumor Size	
< 3 cm	161 (54%)
≥ 3 cm	133 (44.6%)
Unknown	4 (1.4%)

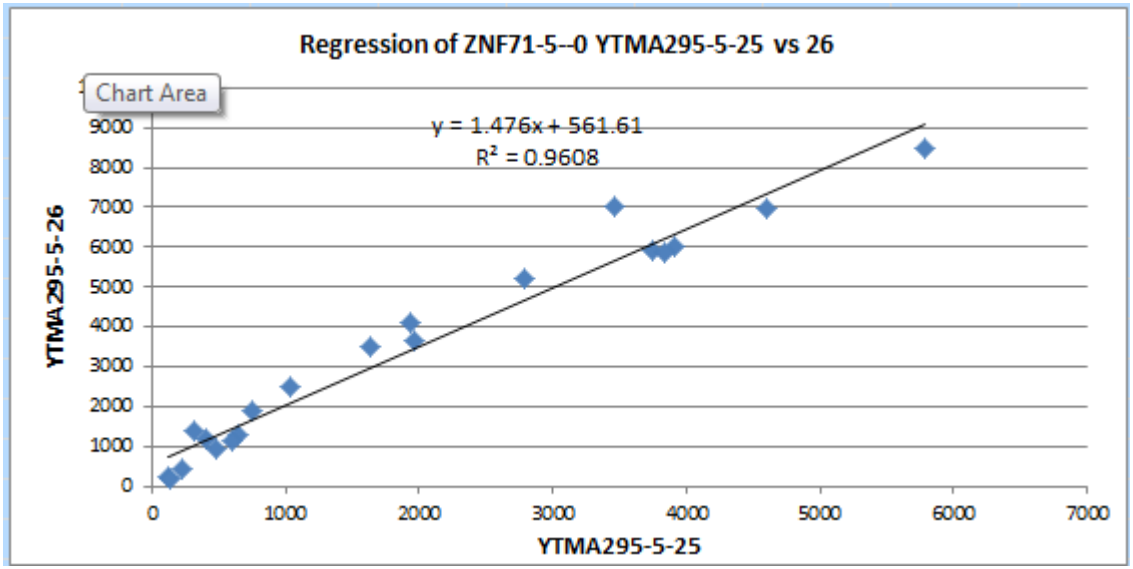
Quantitative expression range graph for titer optimization



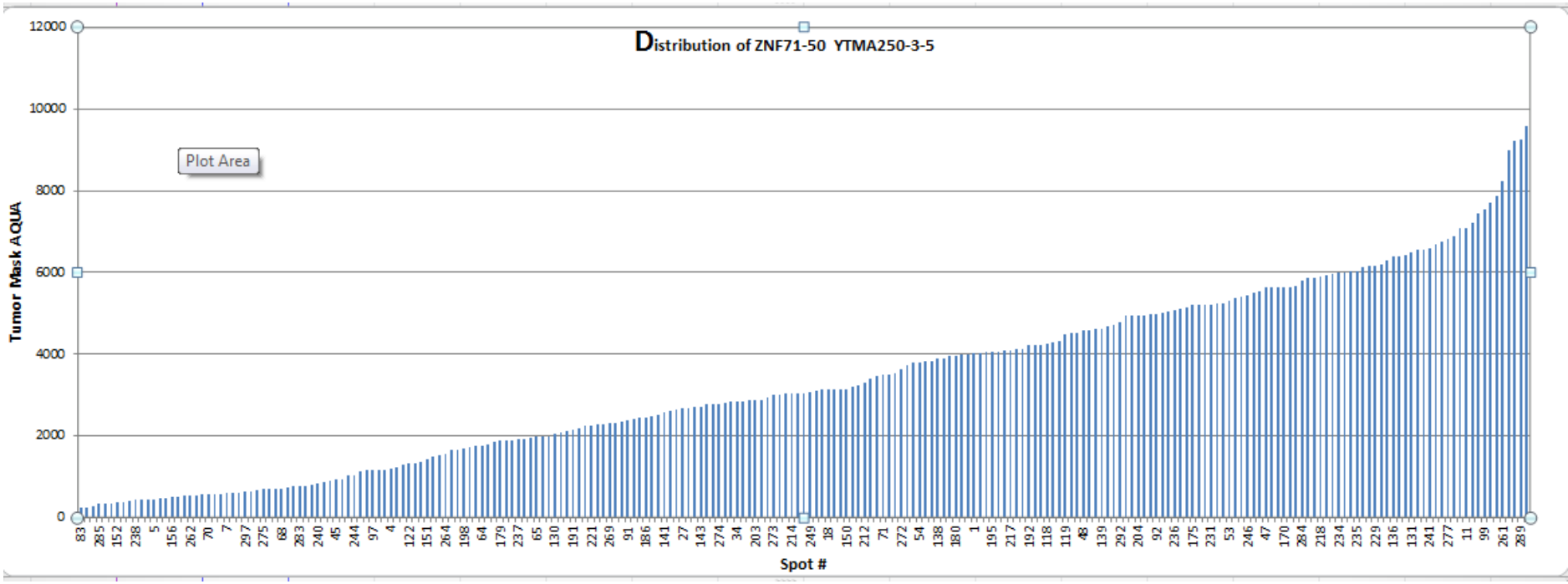
Western Blot for ZNF71



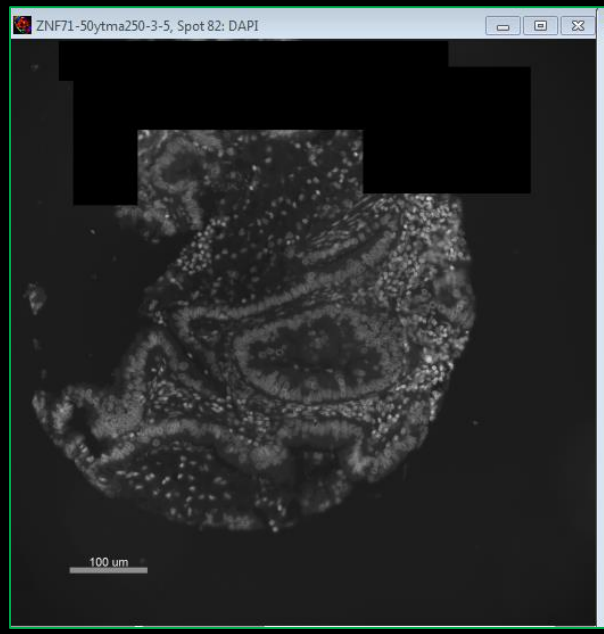
Assay reproducibility for ZNF71

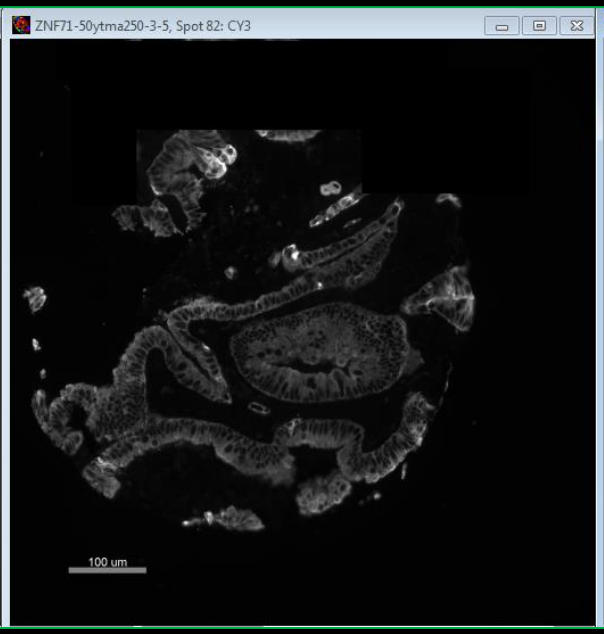


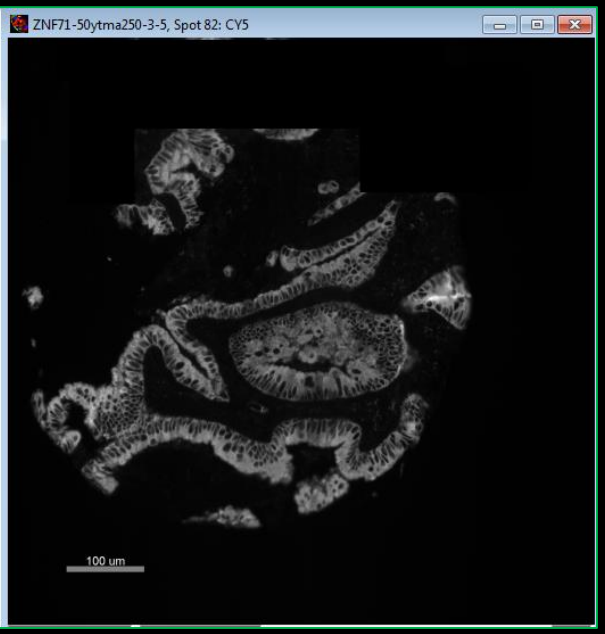
Distribution of ZNF71 scores on NSCLC cohort

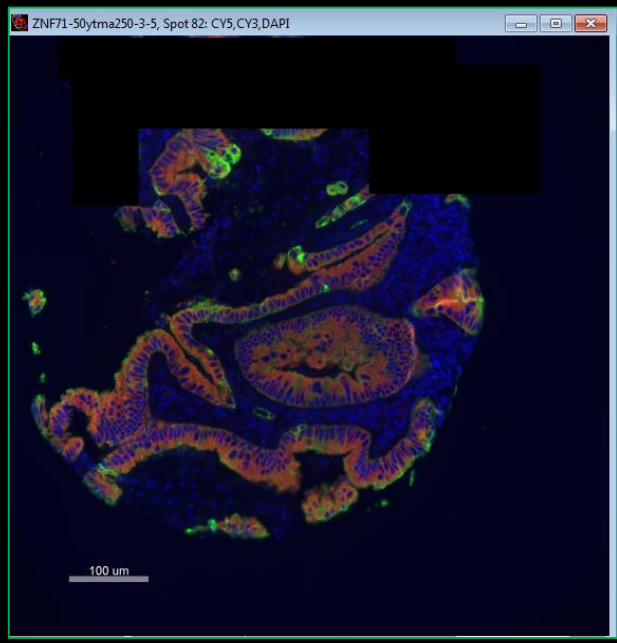


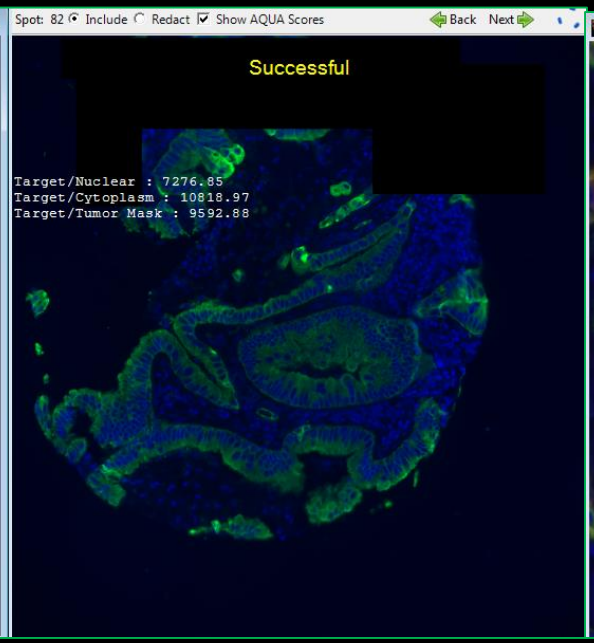
ZNF71 High Expression YTMA250-3-5 Spot# 82

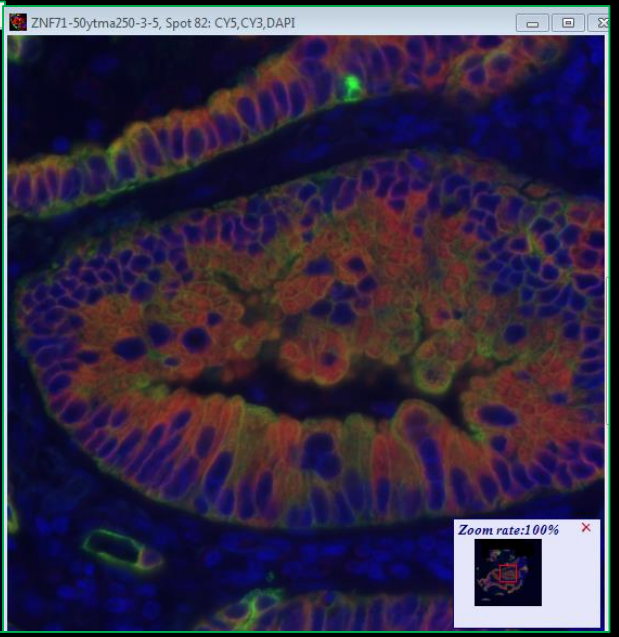




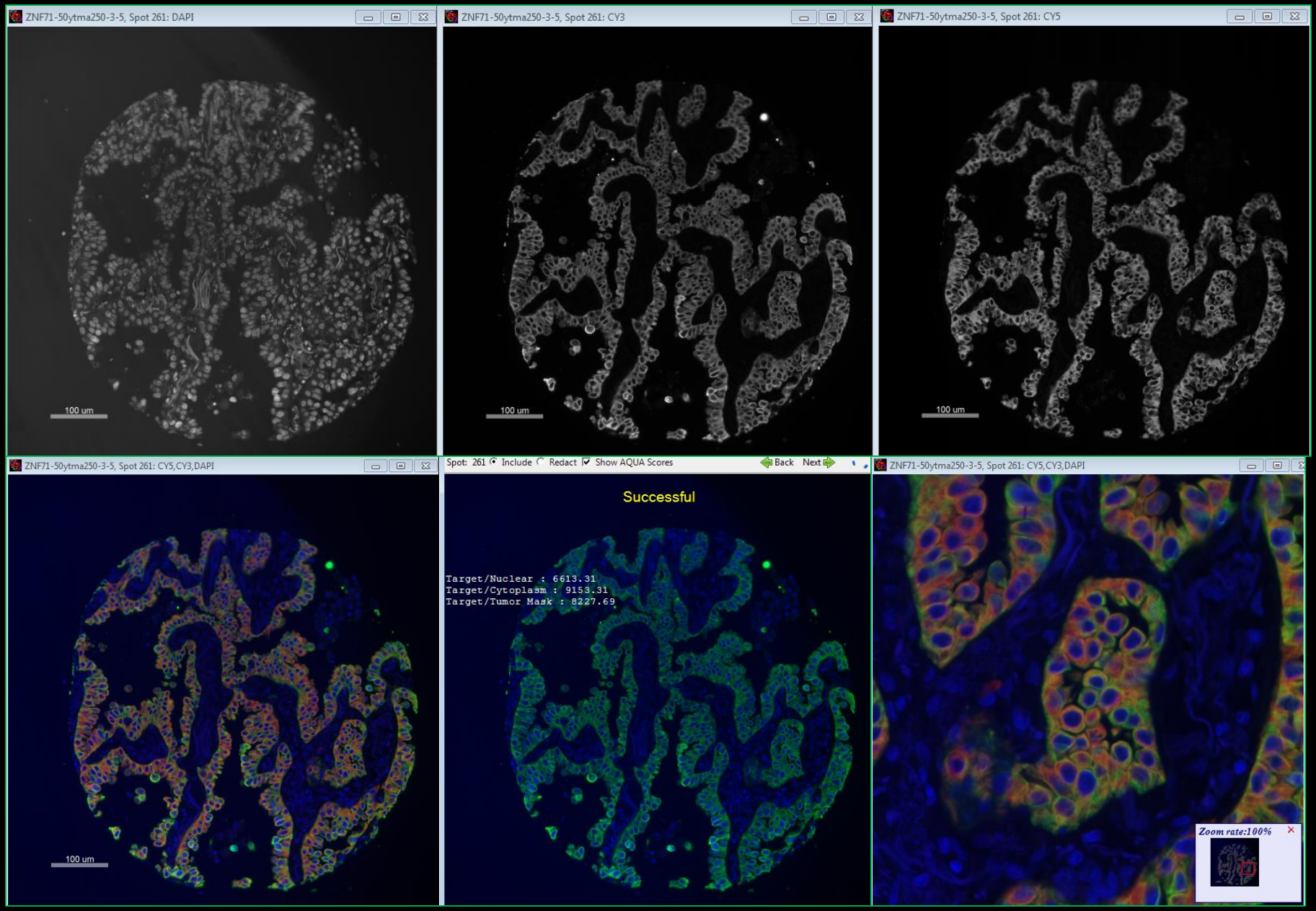




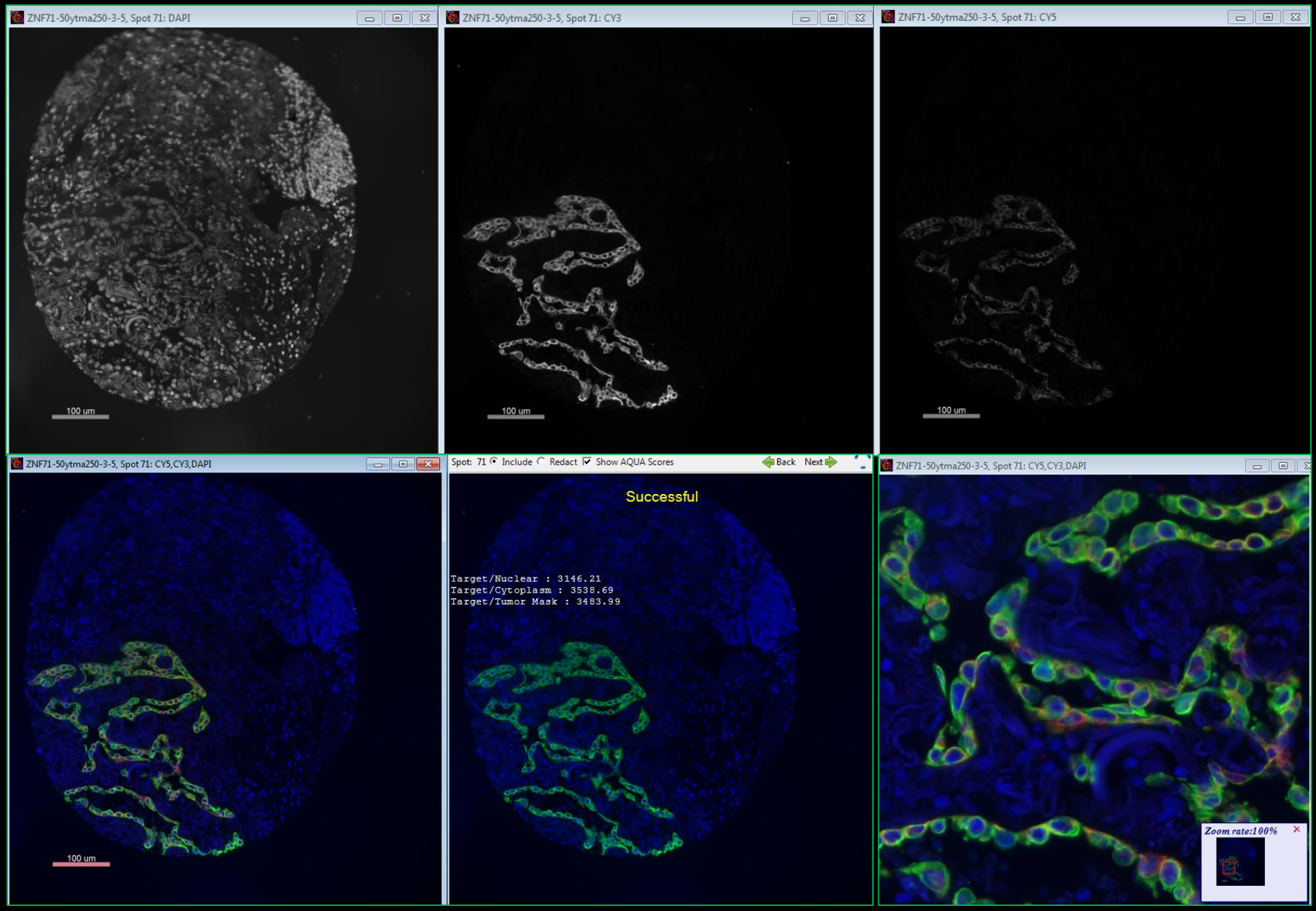




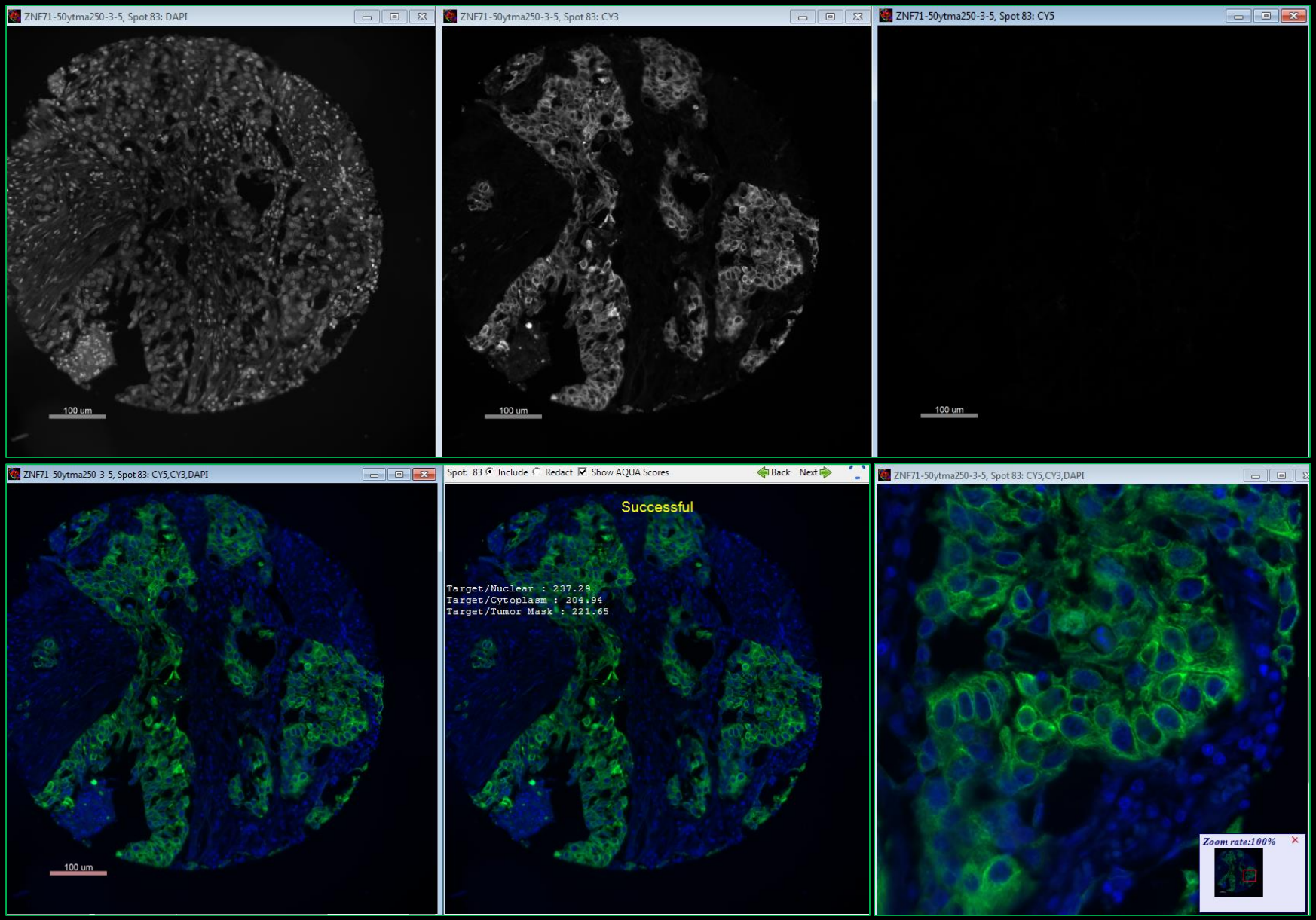
ZNF71 High Expression YTMA250-3-5 Spot# 261



ZNF71 Medium Expression YTMA250-3-5 Spot# 71



ZNF71 Low Expression YTMA250-3-5 Spot# 83



Summary and conclusions

- After optimizing assay conditions and testing reproducibility ZNF71 was stained and quantified on the Yale NSCLC cohort YTMA 250-3-5 using the AQUA method of QIF.

- The data sheet containing clinico-pathological information, follow up and expression levels for ZNF71 is attached to the report for further analysis.

ZNF71 on second cohort of NSCLC cases YTMA 79

STS lab
Yale University, School of Medicine
May 2016

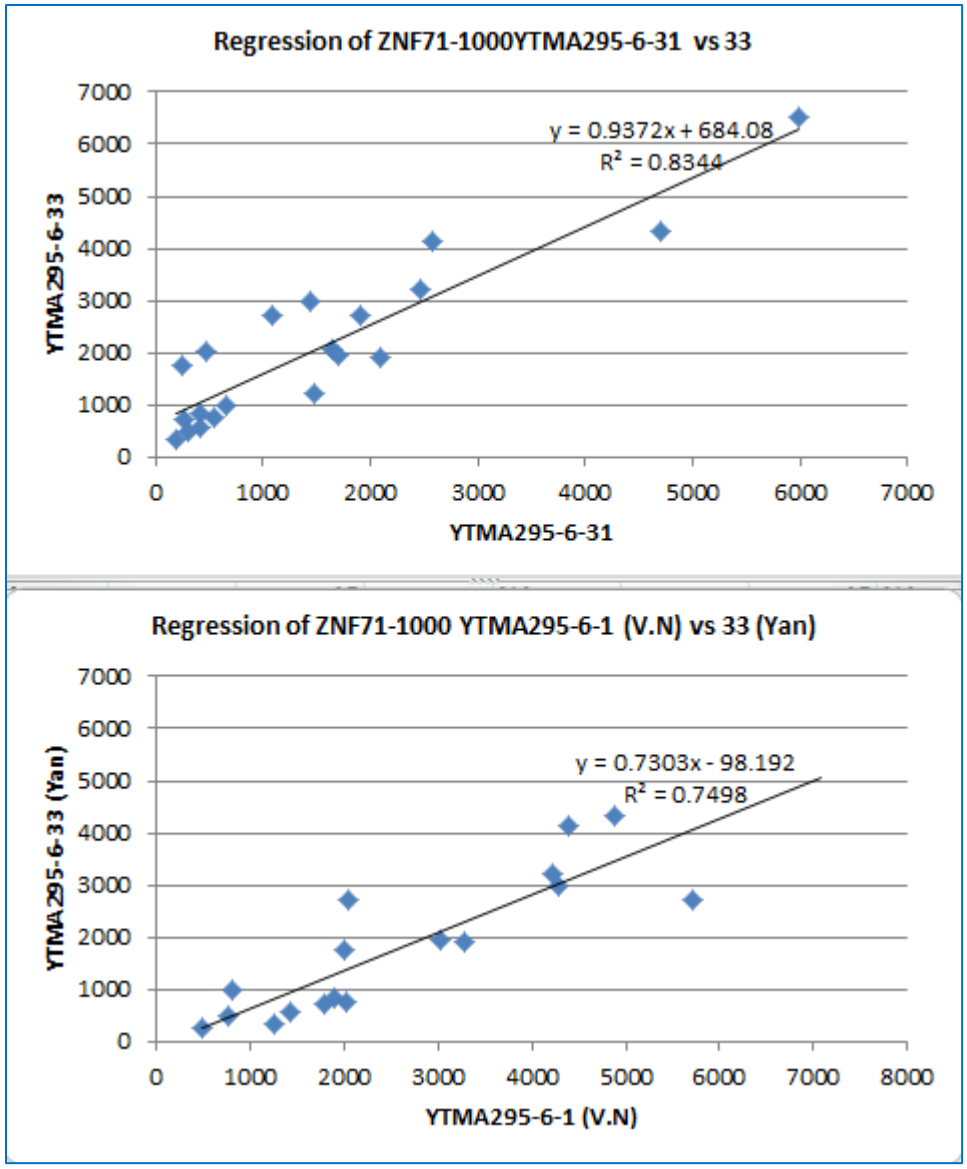
OBJECTIVES and METHODS

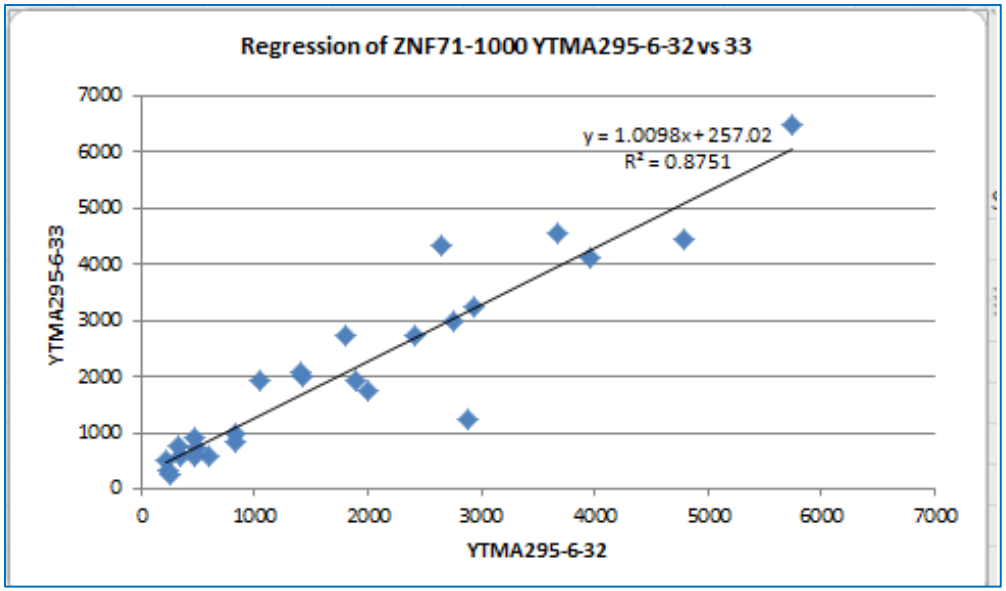
- Quantitative evaluation of expression levels of ZNF71 on a second cohort of NSCLC cases with follow up data using the AQUA method of QIF
- The antibody was previously optimized.
- A new ZNF71 antibody was purchased and re-titrated. Results were compared to previous stains.
- YTMA 79 was stained and quantified.

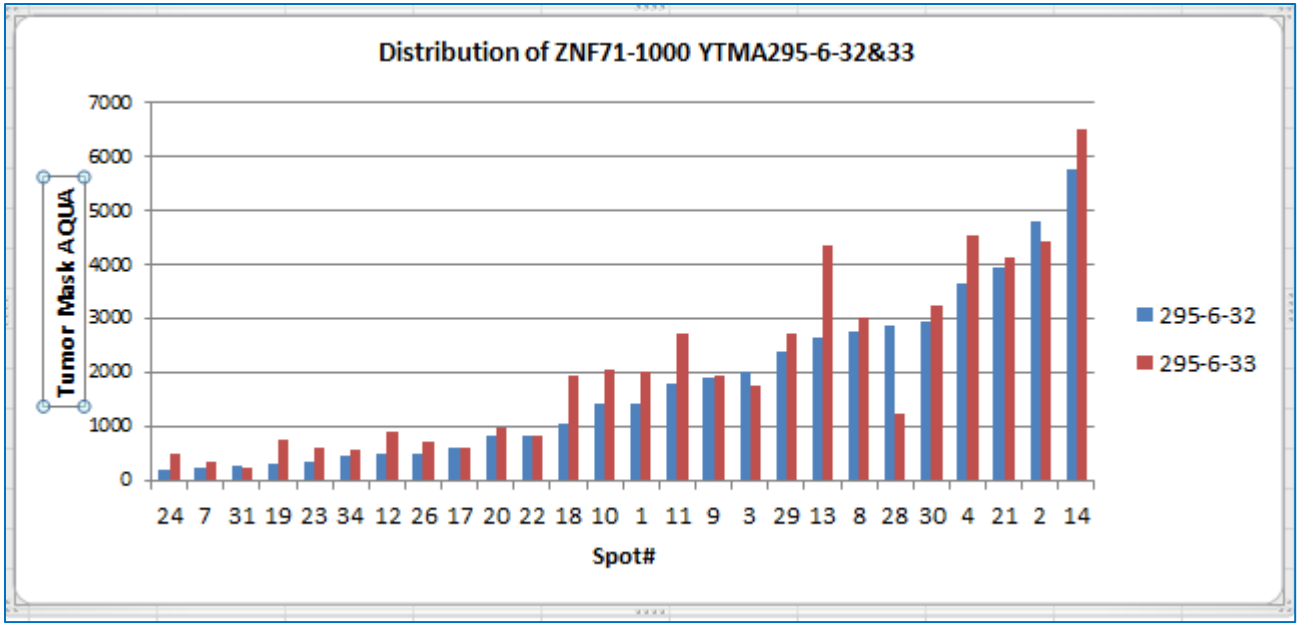
COHORT INFORMATION

Parameter	YTMA79
All Patients (%)	202 (100%)
Date of diagnosis	1988-2003
Age (Years)	
< 70	129 (63.9%)
≥ 70	73 (36.1%)
Gender	
Male	96 (47.5%)
Female	106 (52.5%)
Smoking Status	
Never	14 (6.9%)
Smoker	185 (91.6%)
Unknown	3 (1.5%)
Histology	
Adenocarcinoma	116 (57.4%)
Squamous	33 (16.3%)
Other	54 (26.3%)
Stage	
I and II	126 (62.4%)
III and IV	59 (29.2%)
Unknown	17 (8.4%)
Primary Tumor Size	
< 3 cm	80 (39.6%)
≥ 3 cm	59 (29.2%)
Unknown	63 (31.2%)

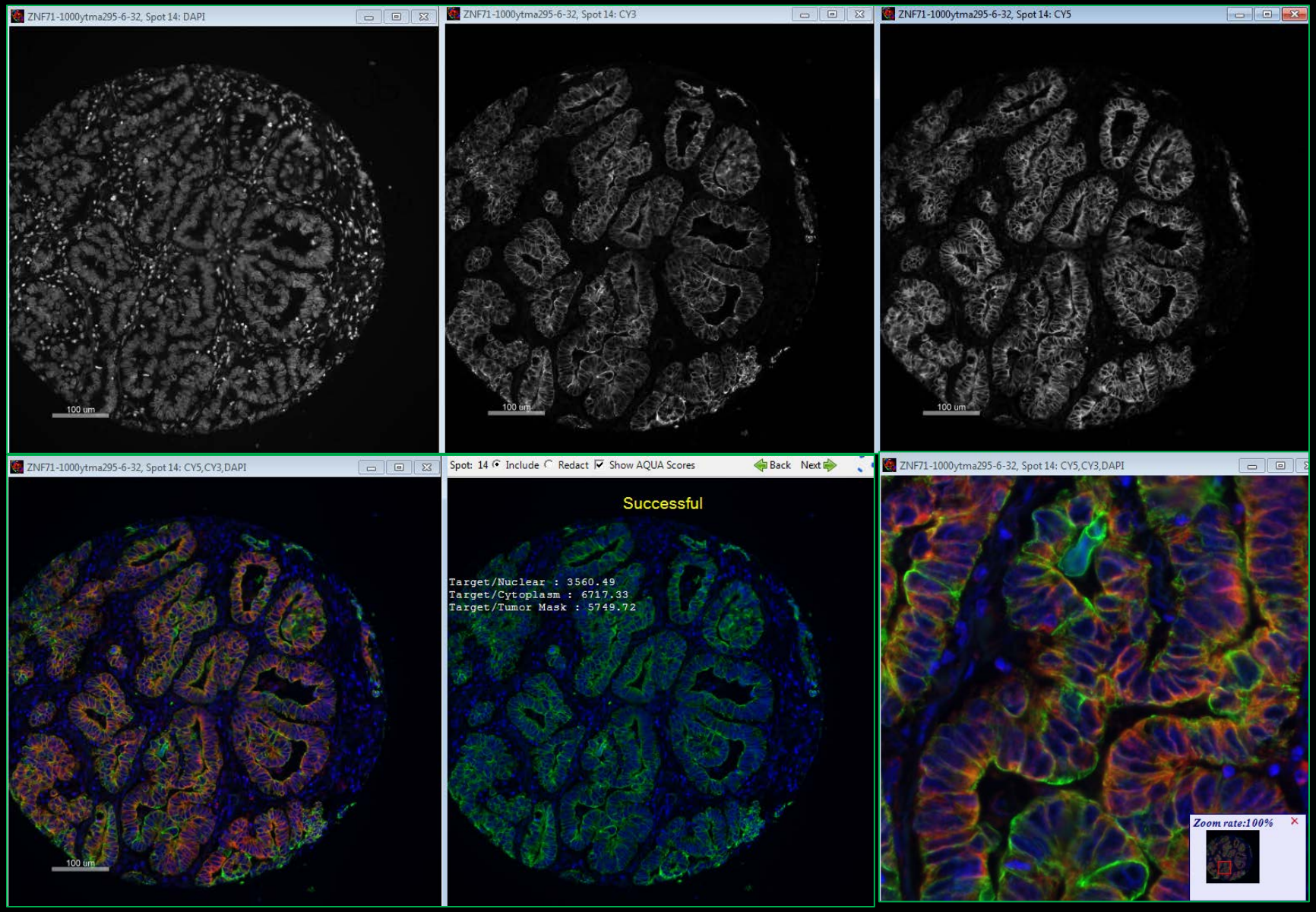
ASSAY REPRODUCIBILTIY



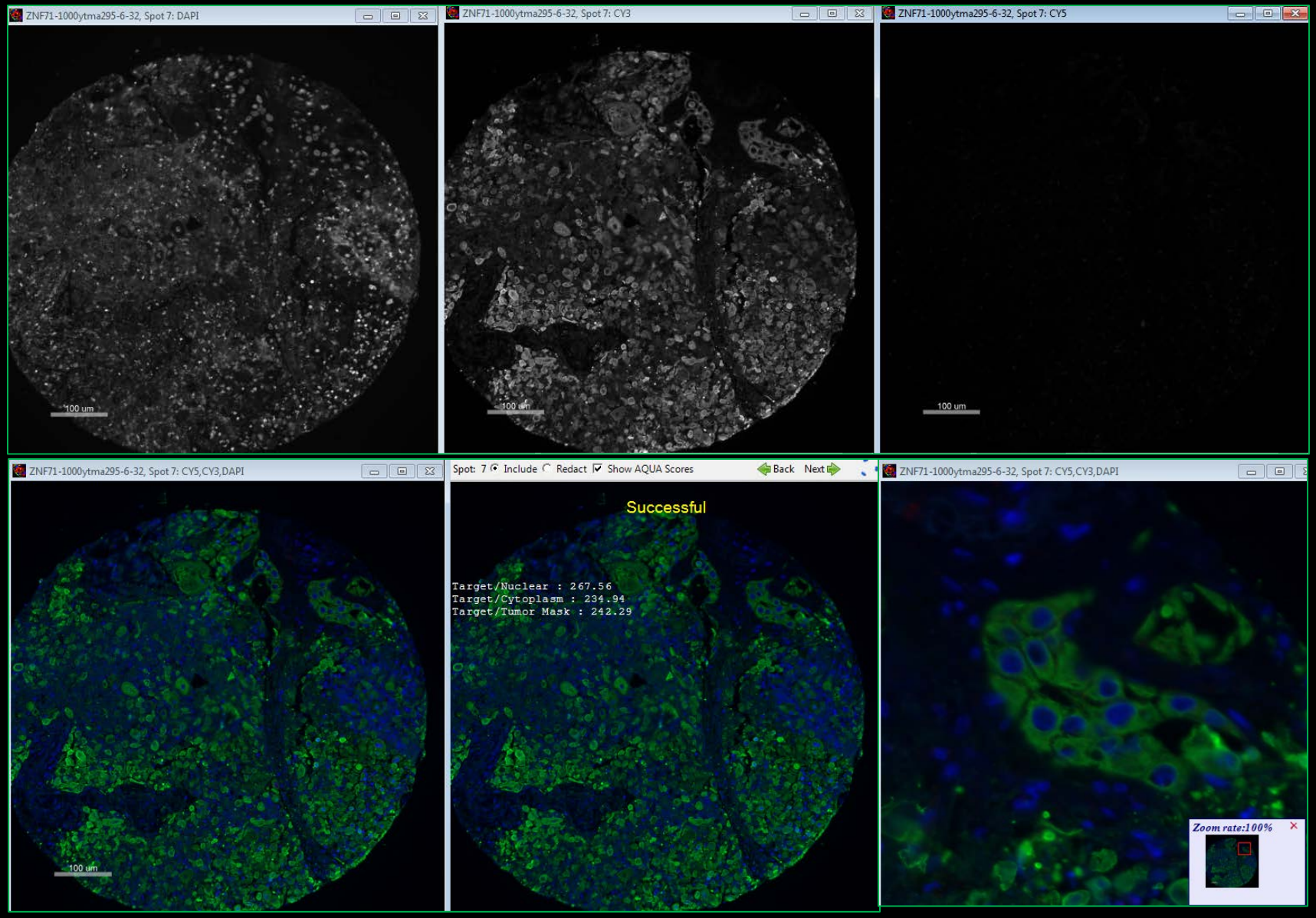

Regression of ZNF71-1000 YTMA295-6-32 vs 33
y = 1.0098x + 257.02
R² = 0.8751
YTMA295-6-33
YTMA295-6-32
0
1000
2000
3000
4000
5000
6000
7000


Distribution of ZNF71-1000 YTMA295-6-32&33
Tumor Mask AQUA
Spot#
295-6-32
295-6-33
0
1000
2000
3000
4000
5000
6000
7000
24 7 31 19 23 34 12 26 17 20 22 18 10 1 11 9 3 29 13 8 28 30 4 21 2 14

ZNF71-1000 YTMA295-6-32 Spot# 14 High Score



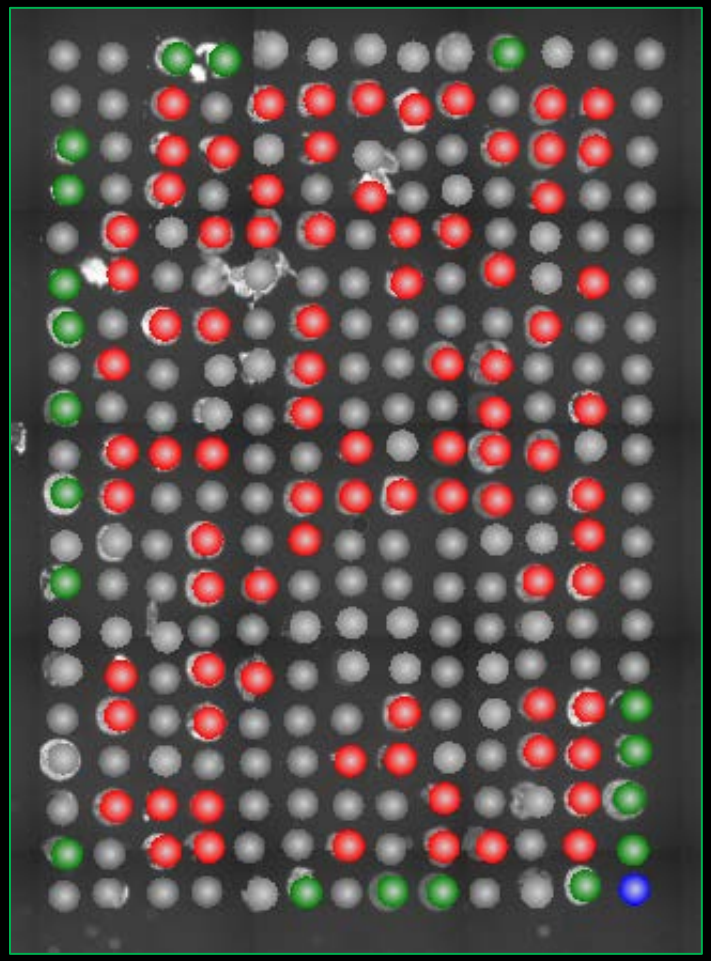
ZNF71-1000 YTMA295-6-32 Spot# 7 Negative Score



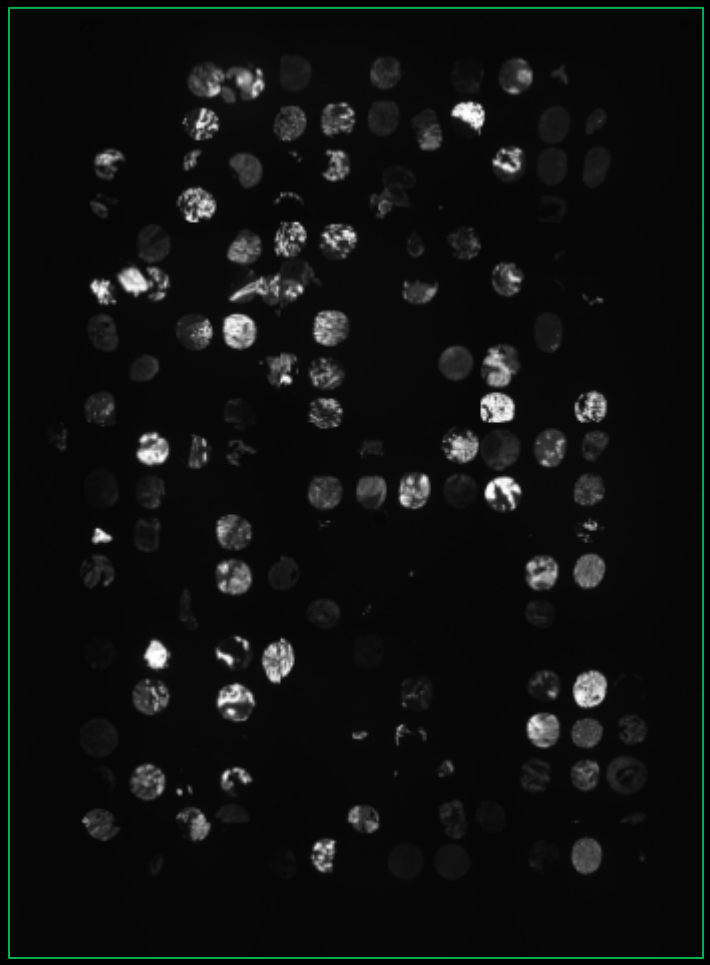
STAINING OF YTMA 79

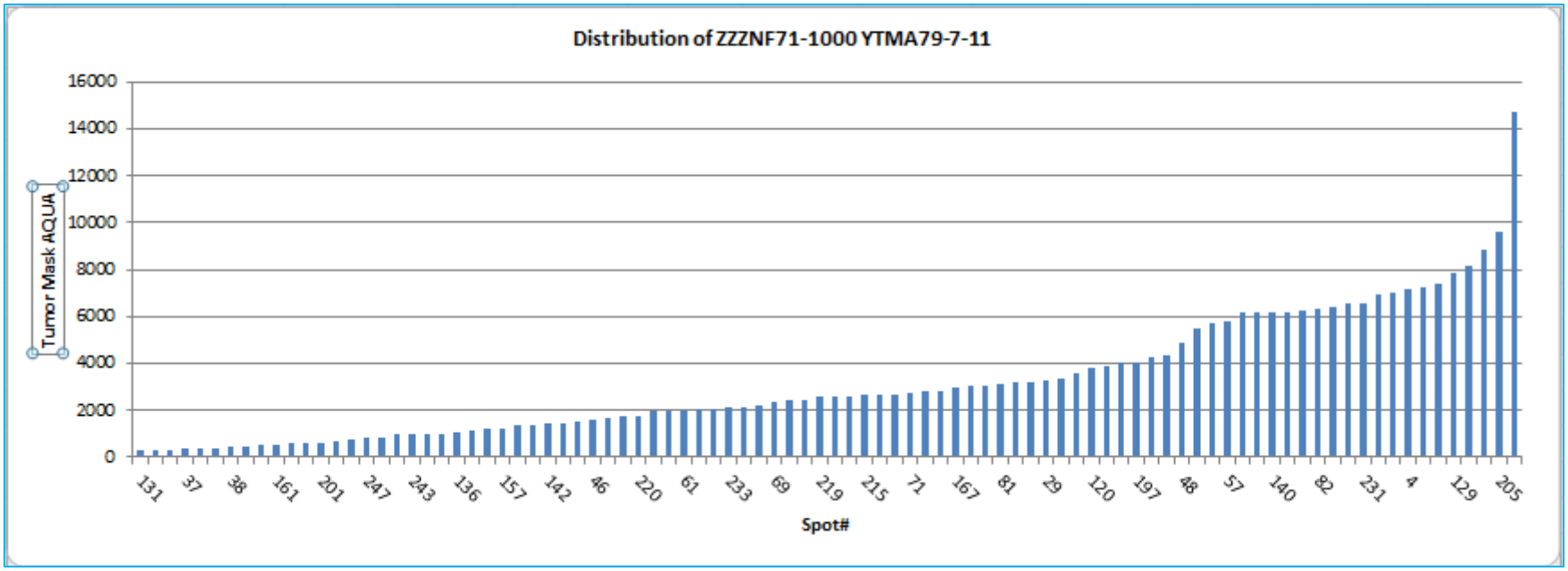
LOW RESOLUTION IMAGE OF YTMA 79

ZNF71-1000 YTMA79-7-11 Map

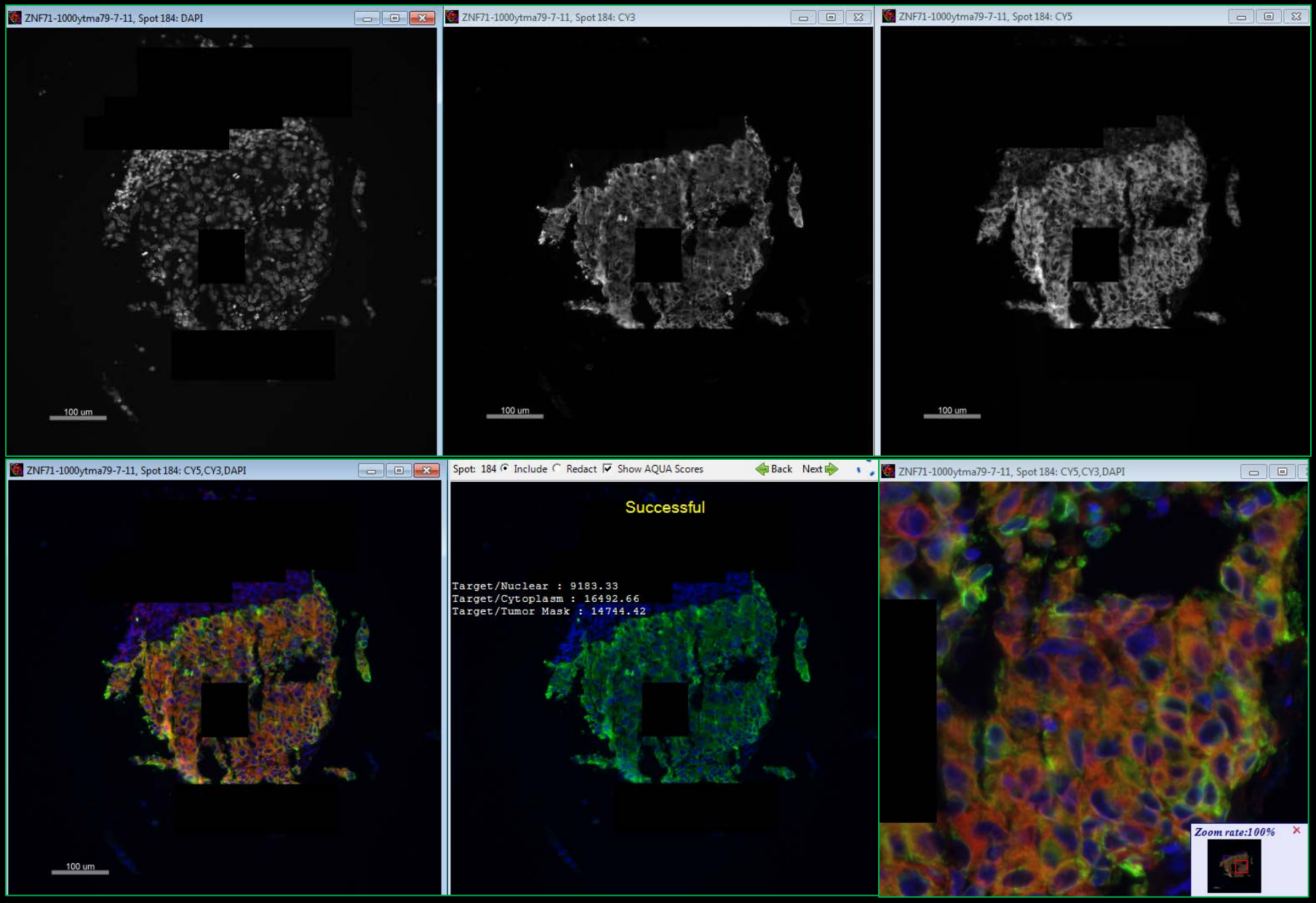


Cy5

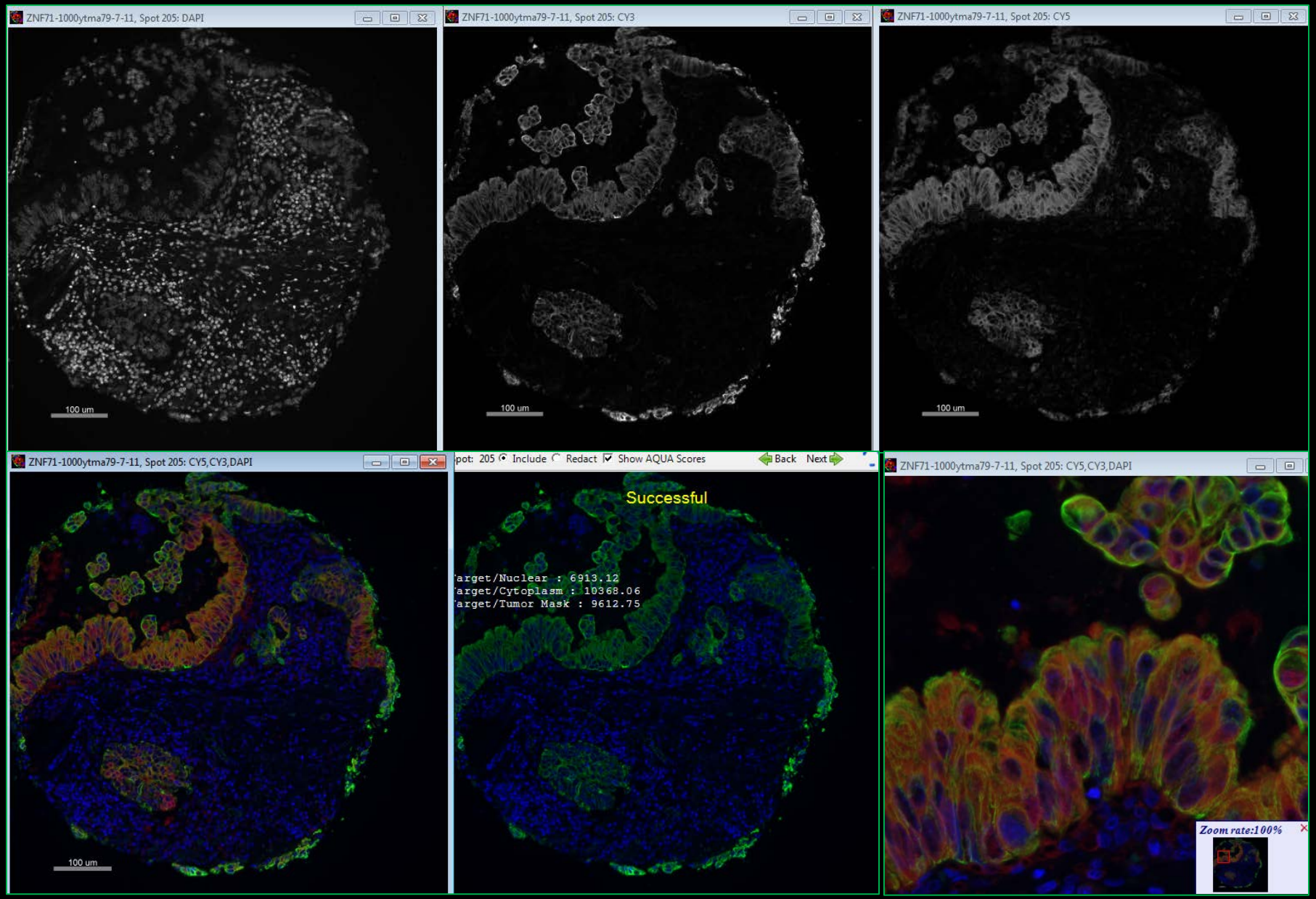



Distribution of ZZZNF71-1000 YTMA79-7-11
Tumor Mask AQUA
16000
14000
12000
10000
8000
6000
4000
2000
0
131
37
38
161
201
247
243
136
157
142
46
220
61
233
69
219
215
71
167
81
29
120
197
48
57
140
82
231
4
129
205
Spot#

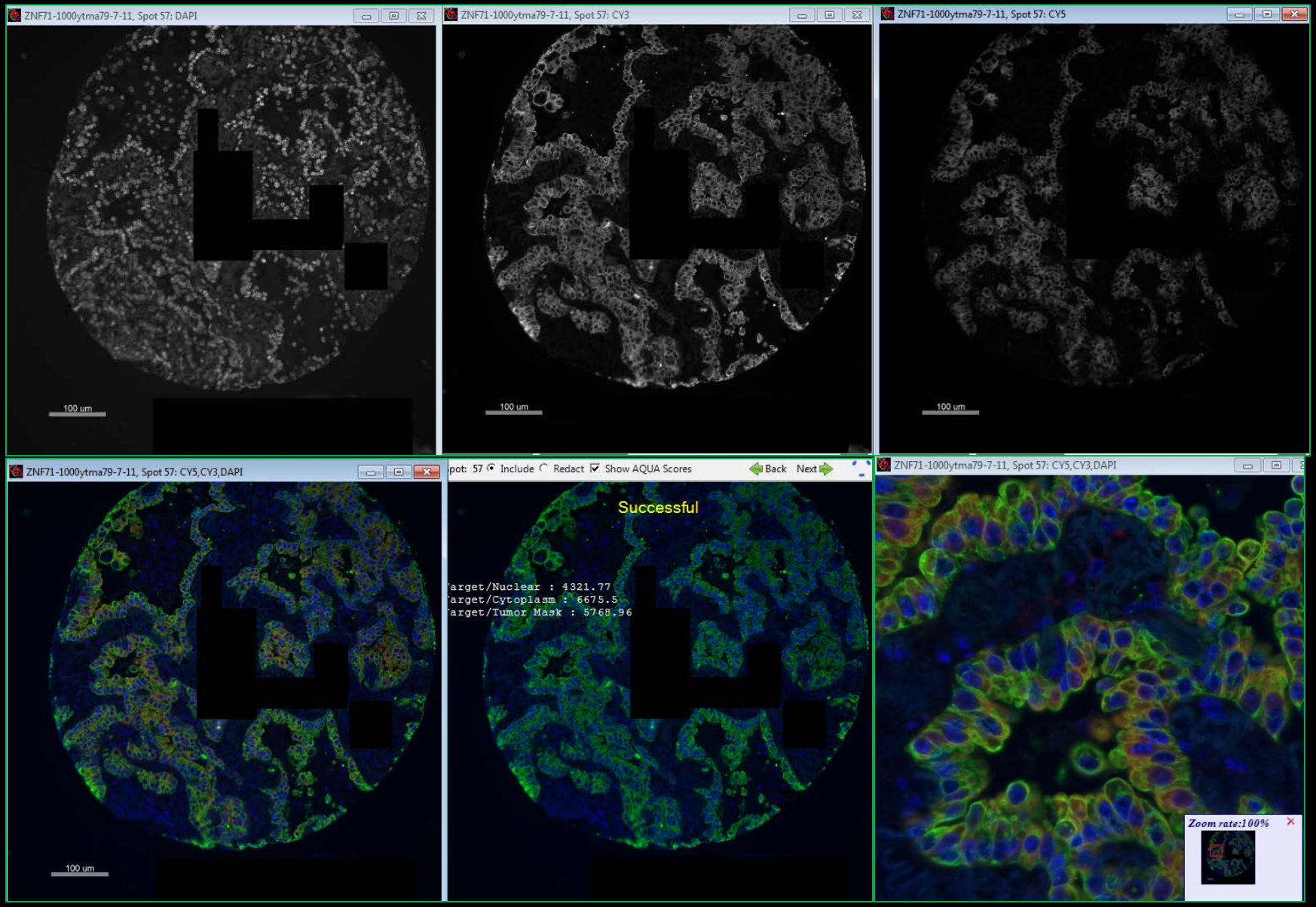
ZNF71-1000 YTMA79-7-11 Spot# 184 High Score



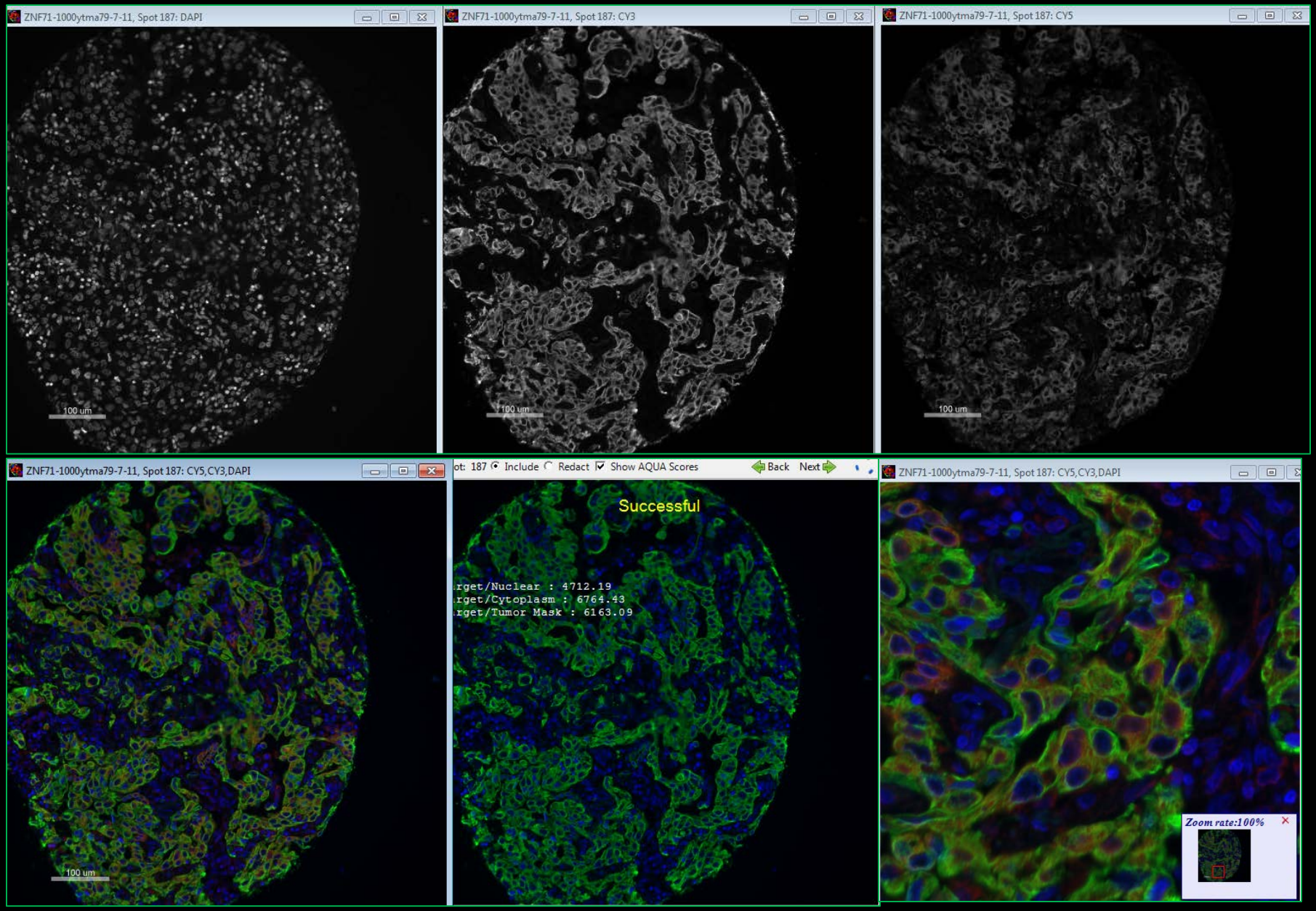
ZNF71-1000 YTMA79-7-11 Spot# 205 High Score



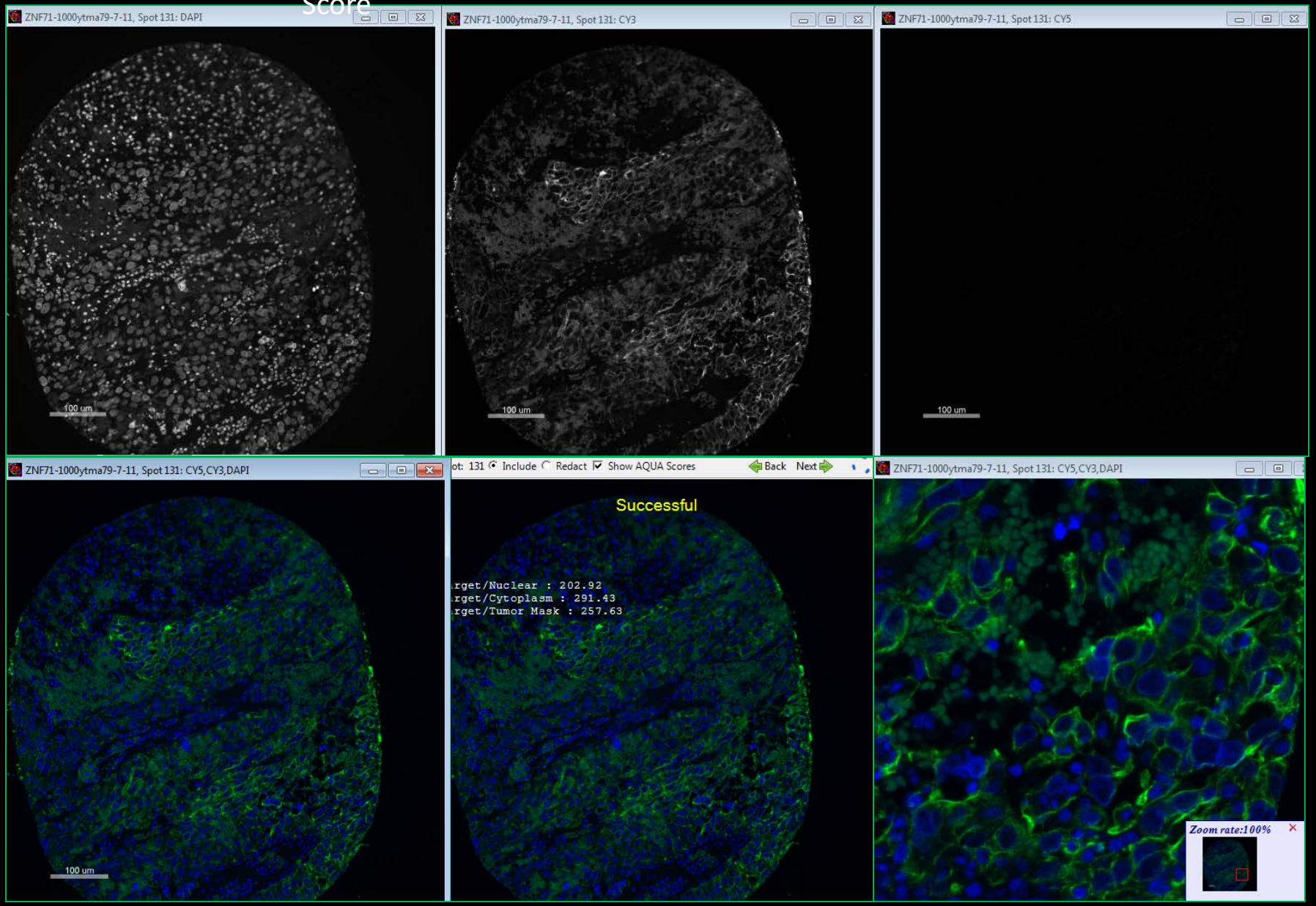
ZNF71-1000 YTMA79-7-11 Spot# 57 Medium Score



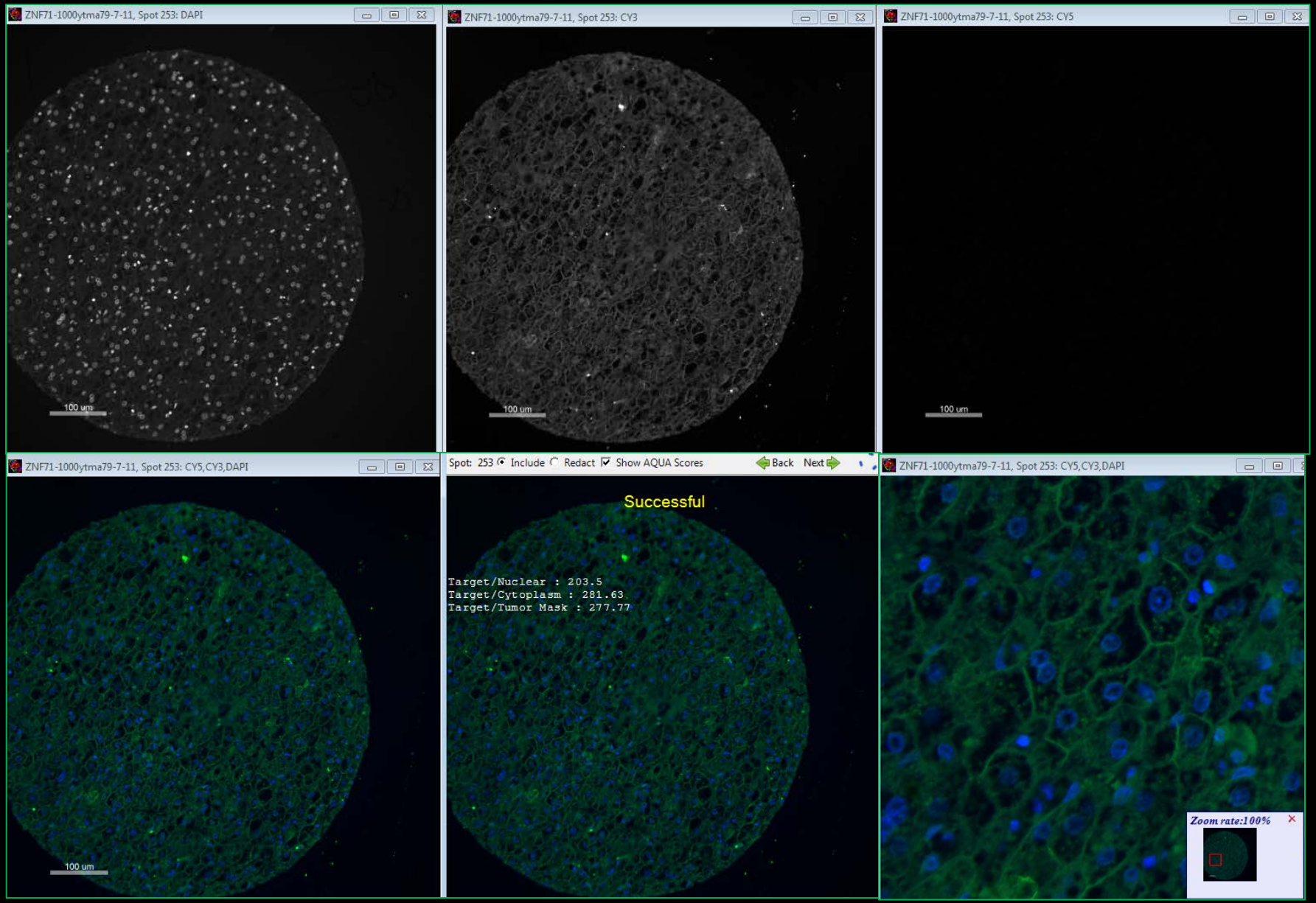
ZNF71-1000 YTMA79-7-11 Spot# 187 Medium Score



ZNF71-1000 YTMA79-7-11 Spot# 131 Negative Score



ZNF71-1000 YTMA 79-7-11 Spot# 253 Negative Score



SUMMARY AND CONCLUSIONS

- A new ZNF71 antibody was purchased and re-titrated.
- The staining conditions and assays regress well to previous results.
- The NSCLC cohort YTMA 79 was stained and quantified for ZNF 71.
- Representative images are shown in this ppt.
- Data sheet for this cohort with follow up information is provided with this report.