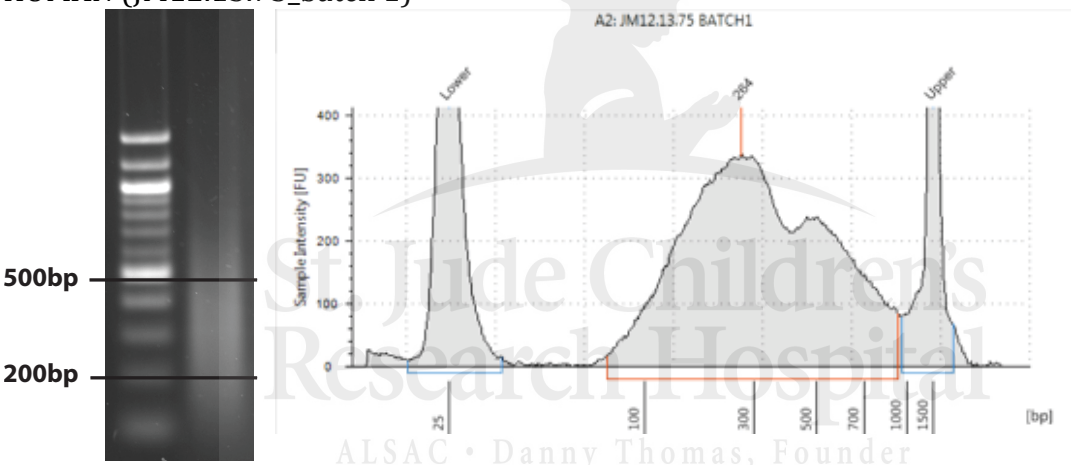
Date: 03/06/14 Experiment #: ID.1.14.27
Antibody code; 15 cat.# 39133

H3K27ac

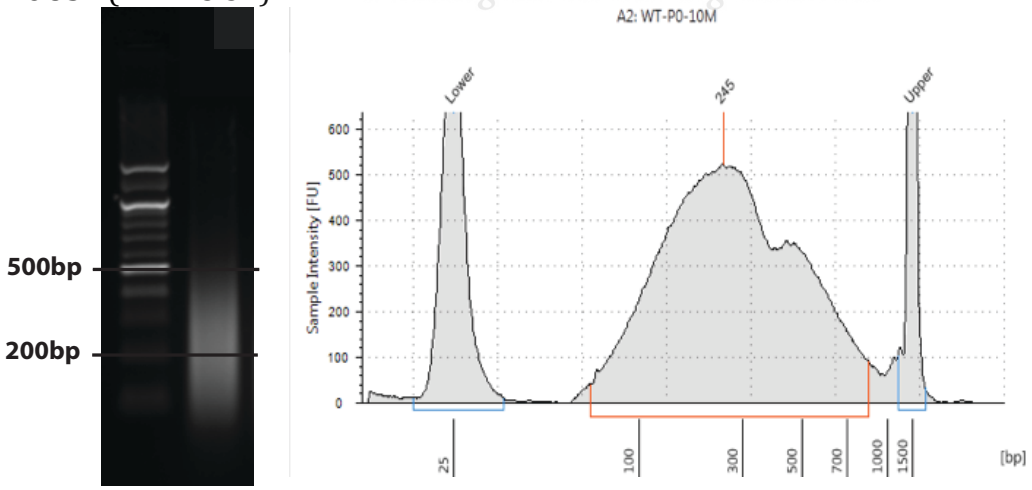
Antibody cat #:	39133
Lot #:	01613007
Company:	Active Motif
Host species:	Rabbit
Date validated:	03/06/2014
Validated by Dyer lab member:	Issam Al Diri
ChIP validated by other:	

Dilution for immunoblot:	1:1000
Antibody amount per ChIP:	4µl/1 million human and mouse cells
Number of cells per ChIP:	
Chromatin prep protocol #:	JM12.14.109 (human); ID.1.13.31 (mouse)
Quant-it for human chromatin:	162ng/µl
Quant-it for mouse chromatin:	77ng/µl
Protein concentration-human:	0.5µg/µl
Protein concentration-mouse:	0.3µg/µl

HUMAN (JM12.13.75_batch 1)



MOUSE (ID.1.13.31)



Date: 03/06/14 Experiment #: ID.1.14.27
Antibody code; 15 cat.# 39133

Level 1: Western Optimization

Purpose:
To test the efficiency and specificity of the antibody in human and mouse cells. This antibody will be used for future IP validation and /or ChIP experiments.

Experiment summary:
5-10 ug of chromatin from human and mouse will be run on a western and immunblotted using the antibody being tested. On the same gel, run the same samples to be immunoblotted for total histone. This will be used for later quantitation.

Procedure: 1-2 days

1) Dilute 28µl of chromatin with 7µl of 5X sample buffer (must add in beta-mercaptoethanol to buffer first) .

2) Heat sample to 95°C for 10 minutes

3) Prepare running buffer and pour into electrophoresis chamber. Place precast gradient gels into the chamber (Biorad TGX 15-well 4-20% (456-1096)). * Remember to remove green strip on the bottom of the gel.

4) Remove comb and rinse out the wells with running buffer.

5) Load 15ul of sample per well. **Remember to run enough lanes to immunoblot for total H3 or total H4.

6) Run gel at 200V (constant volts) for 25 minutes. At this point watch the blue running dye. Continue to let the gel run until the blue dye has reached the bottom. Stop and remove the gel once the blue dye has reached the bottom.

7) Separate the plates and manually remove the blue dye with a razor blade.

8) Place the gel in transfer buffer for 5-10 minutes to equilibrate.

9) Transfer the protein onto 0.2µm nitrocellulose membrane at 100V (constant volts) for one hour.

10) Remove the membrane from transfer apparatus directly into 5mls of Odyssey blocking buffer.

11) Block for one hour. At this point make antibody dilutions using Odyssey blocking buffer. **Remember to immunoblot for total H3 or total H4 for each western. This is important to relatively quantitate each of the H3 marks.

12) Transfer membrane to antibody dilutions and rock over night at 4°C.

13) Wash membrane for 5min with 1% TBST (can find recipe in Dyer lab protocol book). Repeat wash two more times.

14) Transfer membrane to secondary antibody (infrared fluorescent secondary antibody diluted 1:10,000 in Odyssey blocker).

15) Incubate the membrane for 1 hour (covered with foil) at room temperature on the Belly Dancer.

16) Wash membrane for 5min with 1% TBST (can find recipe in Dyer lab protocol book). Repeat wash two more times.

17) Scan membranes on the Odyssey LiCor.

18) Determine the signal for each band using LiCor (please ask Lyra or Justina if you need help).

19) Determine relative amounts of the protein by normalizing to total H3 or H4.

Recipes and antibody dilutions:

RUNNING BUFFER (1 liter)

25mM Tris	3.03g
192mM glycine	14.4g
0.1% SDS	5mls (20%)
Up to 1 L with H2O	

TRANSFER BUFFER (1 liter)

25mM Tris	3.03g
192mM glycine	14.4g
methanol	200mls
Up to 1 L with H2O	

Primary Antibody	Cat. #	Species	Dilution or µicrograms	µl to add to 5mls of Oddyssey blocker
H3K27ac	39133	Rabbit	1:1000	5µl
Total H3	Ab10799	Mouse	1:1000	5µl
Secondary Antibody	Cat. #	Species	Dilution or µicrograms	µl to add to 5mls of Oddyssey blocker
IRDYE680	926-68071	Rabbit	1:10,000	0.5µl
IRDYE800CW	926-32210	Mouse	1:10,000	0.5µl

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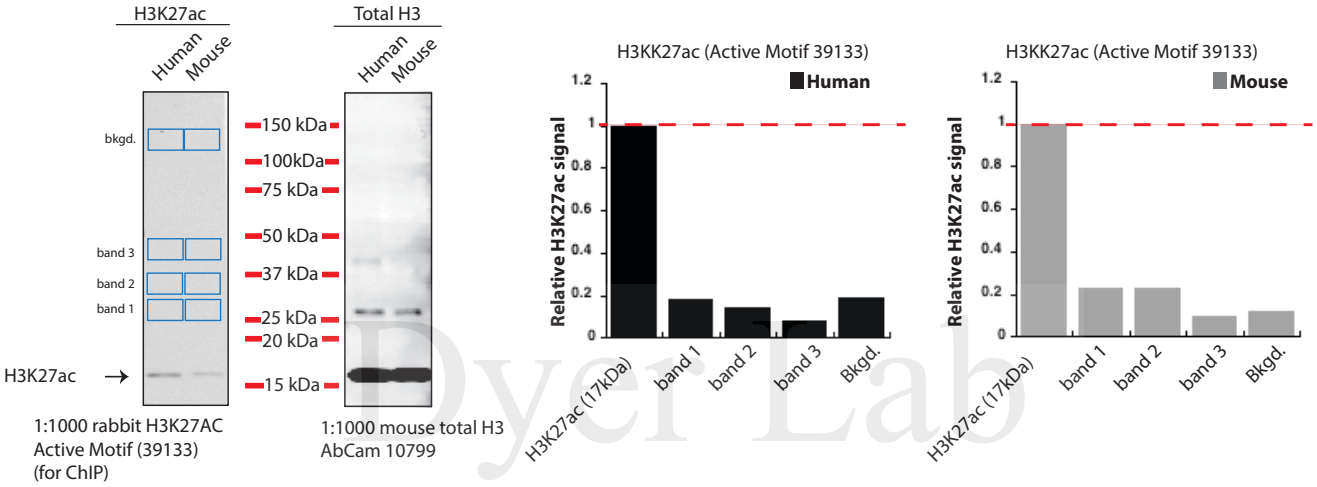
Results:

Total protein of human chromatin loaded per lane: 3µg
Total protein of mouse chromatin loaded per lane: 3µg



Species	Band	Relative to H3K27ac
Human	H3K27ac (17 kDa)	1.000
	Band 1	0.180
	Band 2	0.145
	Band 3	0.078
	Bkgd.	0.186
Mouse	H3K27ac (17 kDa)	1.000
	Band 1	0.225
	Band 2	0.228
	Band 3	0.095
	Bkgd.	0.122

Conclusions: (provide yes or no answer)

The background for this antibody in human is acceptable for use in ChIP: YES
Antibody passes validation for the level 1 for ***human***: YES

The background for this antibody in mouse is acceptable for use in ChIP: YES
Antibody passes validation for the level 1 for ***mouse***: YES

Additional notes:

The major proportion (> 59%) of H3K27ac is at the predicted size of 17 kda. This is an acceptable signal for the expected band according to ENCODE guidelines where it is recommended that the primary reactive band represents >50% of the signal.

Date: 03/06/14 Experiment #: ID.1.14.27
Antibody code; 15 cat.# 39133

Level 2: ChIP Validation

(proceed if antibody passes level 1)

Purpose:

To determine the optimal chromatin:antibody ratio for ChIP.

Experiment summary:

Test 3 two-fold serial dilutions of human and mouse chromatin starting with 1 million cells per ChIP. Two antibody concentrations will be tested for each chromatin dilution. All ChIPs will be done in triplicate in order to quantitate the amount of protein bound to protein-A beads and to quantitate the amount of chromatin pulled down. IgG negative control will be included in this part of the study. This is a total of 14 IPs per species.

Total number of IPs for level 2 validation for both mouse and human: 14 (double up on ChIP reagents to split for protein bound and DNA purification)

Total amount of chromatin needed per species: 1100µl

Antibody concentration #1 to test: 4 µl
Antibody concentration #2 to test: 2 µl

Mouse chromatin ID: ID.1.13.131
Human chromatin ID: JM.12.14.109

Procedure using Diagen0de iDeal ChIP-seq kit (C01010050):

Protein A Magnetic immunoprecipitation: Day 1 (about 2 hours)

use siliconized 1.5ml eppendorf tubes and filter tips for each step

1. Take the required amount of DiaMag Protein A-coated magnetic beads (20 µl/IP). You will need 600µl.

2. Make a 5,600µl 1x ChIP buffer iC1:
   Dilute 1120µl of 5X iC1 buffer with 4,480µl of ChIP grade water in a 15ml conical tube
   Place the diluted ChIP buffer iC1 on ice.

3. Wash the beads 4 times 1,200 µl of ice-cold 1x ChIP buffer iC1. For each wash, resuspend the beads by pipetting up and down several times and place the tubes in the 1.5 ml magnetic rack. Wait for one minute to allow the beads to be captured by the magnet and remove the supernatant. Repeat this 3 times.

4. After the last wash, resuspend the beads in 600µl 1x ChIP buffer iC1.

5. Prepare the ChIP reaction mix for 28 ChIPs + extra for error in a 15ml conical tube:

# of IP's	5% BSA (µl)	200x Protease inhibitor cocktail (µl)	5x buffer iC1 (µl)	Protein A Magnetic beads (µl)	ChIP-seq grade water (µl)
30	180	45	1680	600	3495

6. **IMPORTANT*** Remove 820µl and place in a separate tube. This is for IgG.

7. Aliquot the remaining ChIP working mix into two 15ml conical tubes, one for each antibody concentration being tested. This should be 2,590µl per tube. Next, add in antibody and mix:

Abs. concentration #1: ___4__µl per reaction x 12.5 = __50___µl total to ChIP mix
Abs. concentration #2: ___2__µl per reaction x 12.5 = __25____µl total to ChIP mix

IgG: 2µl per reaction x 4 = 8µl

8. Aliquot 400µl of each antibody working mix into 6 tubes (12 tubes total).

9. Aliquot 400µl of IgG working mix into 2 tubes.

10. Prepare chromatin dilutions. To the starting chromatin, please add 1x Covaris shearing buffer to bring the volume up to 1100µl.

1X Covaris shearing buffer (2mls)
Add 200µl of 10X Covaris shearing buffer (D3) from kit (PN 520075) to 1780µl of cold diH2O plus 20µl of 100X protease inhibitors.
Add 250 ul of D3 to each of the chromatin tubes. Mix thoroughly.

HUMAN	Chromatin (10x10e6 cells/ml)	1X Covaris Shearing buffer	Total volume
1 million cells	700µl	0	700µl
0.5 million cells	250µl	250µl	500µl
0.25 million cells	125µl	375µl	500µl

Mouse	Chromatin (10x10e6 cells/ml)	1X Covaris Shearing buffer	Total volume
1 million cells	700µl	0	700µl
0.5 million cells	250µl	250µl	500µl
0.25 million cells	125µl	375µl	500µl

11. Add *HUMAN* chromatin to the appropriate tubes:
****keep leftoever chromatin for input samples****

- ☐ To ***1*** tube of abs. conc. #1 __4_µl, add 200µl of chromatin from ***1 million cells***.
- ☐ To ***1*** tube of abs. conc. #1__4___ µl , add 200µl of chromatin from ***0.5 million cells***.
- ☐ To ***1*** tube of abs. conc. #1 ___4__ µl, add 200µl of chromatin from ***0.25 million cells***.

- ☐ To ***1*** tube of abs. conc. #2 ___2_ µl, add 200µl of chromatin from ***1 million cells***.
- ☐ To ***1*** tube of abs. conc. #2___2__ µl, add 200µl of chromatin from ***0.5 million cells***.
- ☐ To ***1*** tube of abs. conc. #2 __2___ µl, add 200µl of chromatin from ***0.25 million cells***.

- ☐ To ***1*** tube of IgG, add 200µl of chromatin from ***1 million cells***.

Total volume per tube should be 600µl

12. Add *MOUSE* chromatin to the appropriate tubes:
    ****keep leftoever chromatin for input samples****

    ☐ To ***1*** tube of abs. conc. #1 __4__µl, add 200µl of chromatin from ***1 million cells***.
    ☐ To ***1*** tube of abs. conc. #1___4_µl, add 200µl of chromatin from ***0.5 million cells***.
    ☐ To ***1*** tube of abs. conc. #1 __4__µg, add 200µl of chromatin from ***0.25 million cells***.

    ☐ To ***1*** tube of abs. conc. #2 __2__µl, add 200µl of chromatin from ***1 million cells***.
    ☐ To ***1*** tube of abs. conc. #2__2__µl, add 200µl of chromatin from ***0.5 million cells***.
    ☐ To ***1*** tube of abs. conc. #2 __2__µl, add 200µl of chromatin from ***0.25 million cells***.

    ☐ To ***1*** tube of IgG, add 200µl of chromatin from ***1 million cells***.

13. Incubate the tubes overnight at 4°C under constant rotation.

14. Remove 20µl of the remaining chromatin at each dilution and transfer to a new tube. These will be used as the input samples. Place them at 4 degrees overnight. Freeze leftover chromatin in case we need to repeat any input samples.

Elution and Reverse Crosslinking: Day 2 (about 4hours)

15. The next morning, after the overnight incubation, briefly spin the tubes at 1300RPM and place them in the magnetic rack. Wait for one minute and remove the supernatant. Wash the beads with Wash buffer iW1. To wash the beads, add 700 µl of iW1, gently shake the tubes to resuspend the beads and incubate for 5 minutes on a rotating wheel at 4°C.

16. Repeat the wash as described above once with Wash buffer iW2, iW3 and iW4 using the same buffer volume, respectively.

17. While beads are in iW4, remove 350µl from each tube and transfer to new tubes. These will be the duplicate samples that will go on for DNA purification at step 21. Set these aside. Then proceed to step 18 with the remaining samples.

    ****************Stop***************
    For protein bound to beads:

18. Remove the last wash from the duplicate samples (7 human and 7 mouse) and add 65µl of 2X sample buffer and vortex.

19. Prepare the input sample. For 5% input, take 5µl of each input sample and add 60µl of 2X sample buffer and vortex.
20. Incubate at 95C for 10 minutes.

21. Store in -20C until ready to run western.

********Continue with ChIP protocol for the remaining 7 human and 7 mouse ChIPs*******

22. Make the reverse crosslinking mix:
    -Add 8.4ml of iE1 buffer
    -168ul of Proteinase K (20mg/ml - Invitrogen)

23. After removing the last wash buffer, add 400µl of reverse crosslinking mix to the beads.

    Input samples
    Add 390µl of reverse crosslinking mix to10µl of chromatin for each dilution (a total of 6 inputs –human and mouse).

24. Incubate for 30 minutes at 55C in the thermomixer at 1000RPM.

25. Briefly spin the tubes and add 16 µl of iE2 buffer. Wrap parafilm around the lids of the tubes to seal and minimize evaporation. Incubate overnight in a thermomixer at 900rpm at 65°C. Store at -20 O/N.
    If in a rush, it is possible to incubate at 4 hours and still get a good yield.

DNA Purification: Day 3 (about 4hours)
The following protocol is the DNA Purification protocol that was used to establish this validated antibody. A separate protocol that is the current working Dyer protocol for ChIP has been added in the next section.

26. Briefly spin the tubes and place on the magnetic rack. Transfer sample to a new siliconized tube. This step does not include the input samples as they do not have beads.
    Steps 27-29 in the original protocol have been replaced with the following:
    Make a master mix containing:
    2 x 22 = 44 ul of Carrier
    400 x 22 = 8800 ul of isopropanol
    15 x 22 = 330 ul of DNA magnetic beads

27. Add 417 ul of master mix to each tube including input samples.
28. Incubate IP and input samples for 1 hour at room temperature on a rotating wheel .

29. Briefly spin the tubes, place in the magnet rack, wait 1 minute and discard the buffer. Keep the captured beads and add per tube, 100 µl Wash buffer 1. Close the tubes and incubate for 5 minutes at room temperature on a rotating wheel.
    • Do not disturb the captured beads attached to the tube wall.

30. Briefly spin the tubes, place in the magnet rack, wait 1 minute and discard the buffer. Keep the captured beads and add per tube, 100 µl Wash buffer 2. Close the tubes and incubate for 5 minutes at room temperature on a rotating wheel.

- Do not disturb the captured beads attached to the tube wall.

31. Briefly spin the tubes, place in the magnet rack, wait 1 minute and discard the buffer. Keep the captured beads and add 45µl of buffer C (water).

32. Incubate at 55C for 30 minutes in the thermomixer at 1400RPM.

33. Briefly spin the tubes, place in the magnet rack, wait 1 minute and transfer the supernatant to a new 1.5ml siliconized tube. This is your DNA. Quantitate using Quantit or equivalent.

DNA Purification with QIAGEN MinElute PCR Purification kit:
Day 3 (about 3 hours).
This protocol is the current working Dyer protocol for DNA Purification after ChIP. This is not the protocol that was used to validate this antibody, but is recommended for future ChIPs.

34. Add 1.75 mL of buffer PB to each IP, and measure the pH. The pH needs to be 7.0. If you need to adjust the pH, add 3M NaOAC (Can add around 20 uL of 3 M NaOAc to 1.75 mL of buffer PB).

35. Add the reverse crosslinked material to the tube and vortex briefly.

36. Transfer the sample to a Qiagen min-elute column and spin at 1000 x g for 1min. Repeat until all sample has been added, discarding flow-through after each spin.

37. Add 750µl of PE buffer and spin at 1000 x g for 1 min.

38. Remove flow-through and spin at 13,000RPM for 1 min to dry column.

39. Transfer column to a new labeled 1.5 ml tube and add 55 µl of buffer C (from Diagenode kit) .

40. Let the samples sit at RT for 5 min and spin at 13,000RPM for 1 min. This is your DNA.

Quantitative Real Time PCR: Day 3 or4 (about 1 hour)

Positive target primers: human ACTB-2; mouse GAPDH
Negative target primers: human ZNF333; mouse GD Chr6

41. Set up the following quantitative real-time PCR reaction for each primer set:

	1X Mix (µl)	22 X mix human positive	22 X mix human negative	22 X mix mouse positive	22 X mix mouse negative
Sybr green (Select master)	10	220	220	220	220
Primers (10µM)	**1 or 2	44	44	44	44
Water	4	88	88	88	88

If using Active Motif primers, use 2ul instead of 1ul)

42. Load 15µl of PCR reaction mix into at 96 well dish.

43. Add 5µl of reverse crosslinked DNA to each well.

44. Select the "Enrichment PCR" assay on the qPCR:

-5 min at 95C

-40cycles of:
30 sec at 95C
30 sec at 60C
30 sec at 72C

-Add in a melting curve step.

45. Save file as ID.1.14.27_abs15_qpcr

Tapestation: Day 3 or 4 (about 20 minutes)

46. Add 3µl of D1K buffer to 1µl of ChIP DNA into a separate 0.2ml tube

47. Vortex for 5 sec and spin briefly

48. Load the tapestation tape, tubes and tips and run samples according to the manual instructions.

49. Click on the "electrograph" icon and "scale to sample" icon. Then click on edit peaks and select the major peak on the electrograph. Finally, click EPG snapshot. Repeat this step for all samples.

50. Under the file menu, click "create report" and then click "add EPG thumbnails"

51. Save file as ID.1.14.27_abs15_TS

52. If there are some samples where the traces can not be made, please try the HS D1000 reagents for these samples only. Add 2ul of HS D1000 reagent and 2ul of ChIP DNA into a separate 0.2ml tube. Follow the remaining steps. Save file as ID.1.14.27_abs15_TS

Broad Range Quant-it assay: Day 3 or 4 (about 20 minutes)

53. Make a working solution:
    6,965ul of HS buffer
    35ul of HS reagent

54. Add 190ul of working solution to 29 wells of a 96-well black plate.

55. Add 10ul of standard to each well. This should come out to 0ng, 5ng, 10ng, 20ng, 40ng, 60ng, 80ng, 100ng.

56. Add 5ul of sample to each well.

57. Mix all wells by pipetting.

58. Run on plate reader. Choose protocol "picogreen". While on the plate layout, go to template editor and assign the standards and the unknowns (undiluted).

59. Save file as ID.1.14.27_abs15_quantit

Results:

Please copy and paste results. Do not type data into tables

Protein bound to beads:

H3K27Ac bands were not detectable, and therefore were not quantified for this experiment.

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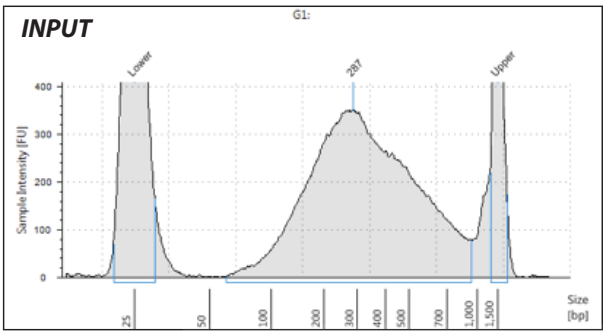
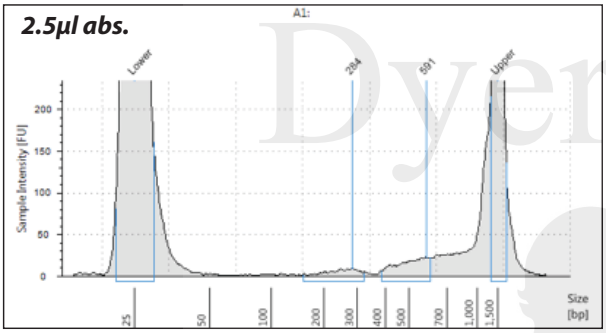
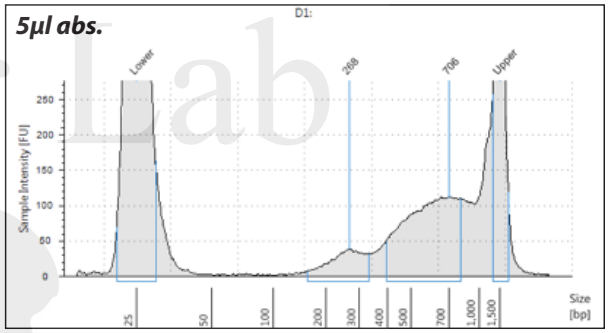
Bioanalyzer of ChIP DNA:

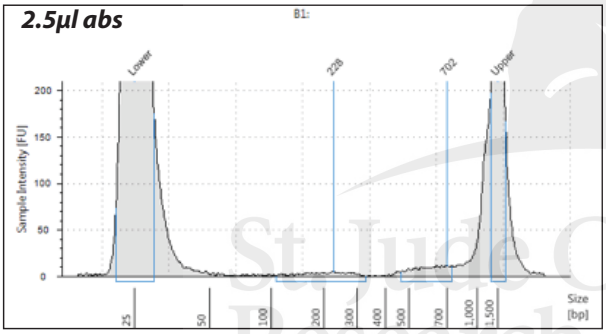
HUMAN

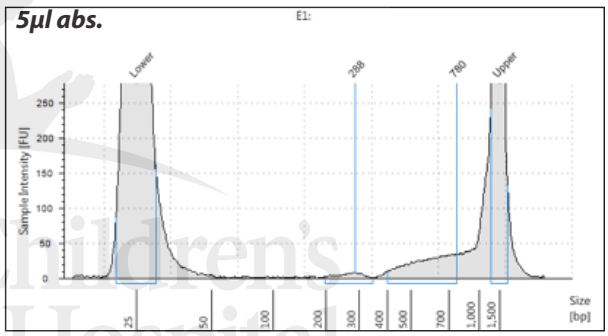




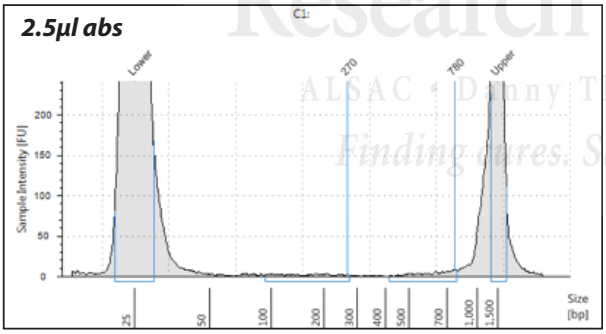


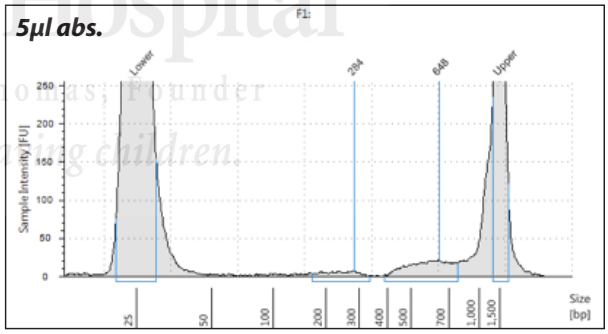
1x10e6 cells





0.5x10e6 cells





0.25x10e6 cells

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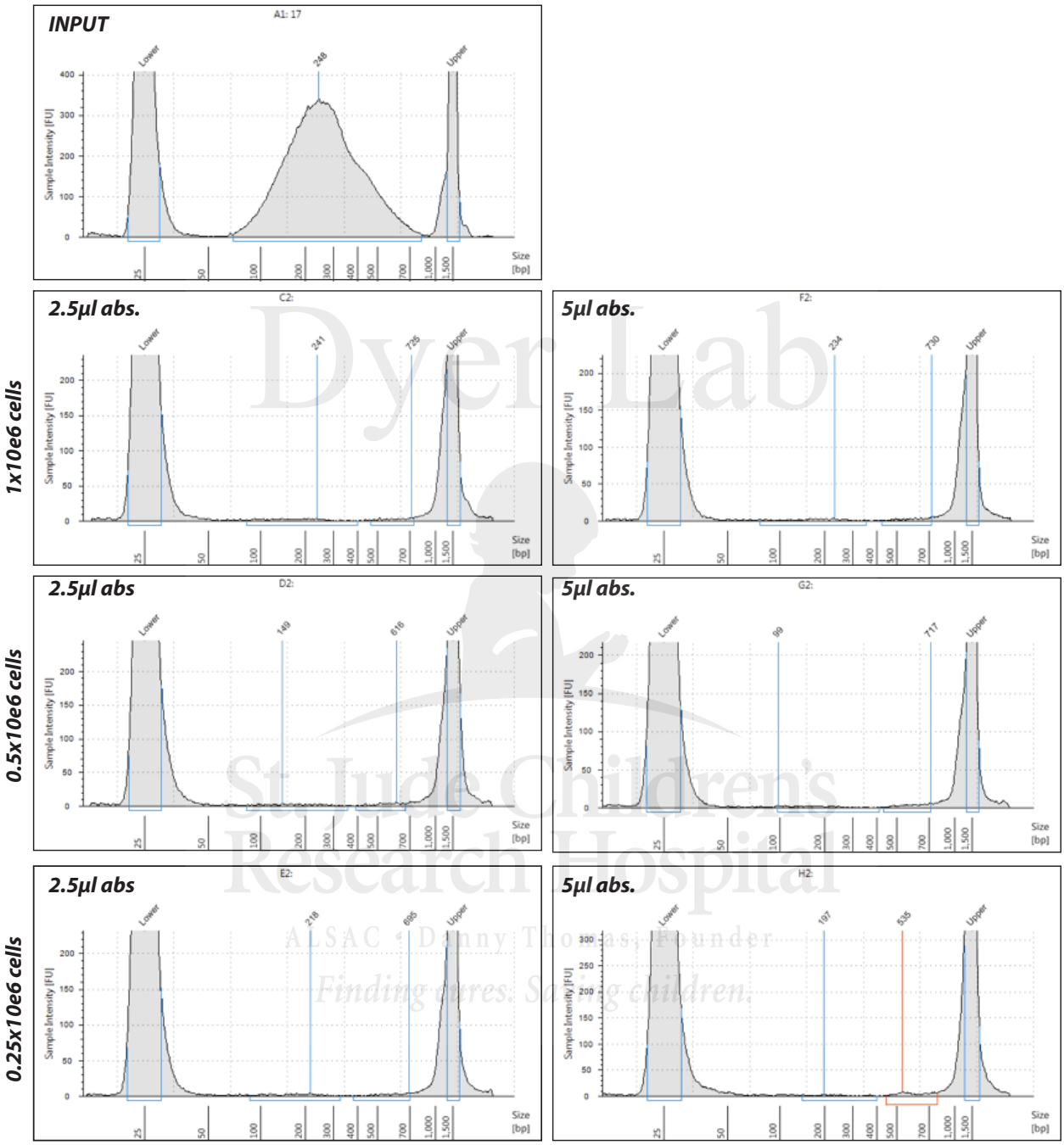
MOUSE



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Quant-it
(*updated from JM12.14.99)

HUMAN	**4µl ab.** (ng/µl)	**Total ng**		**2µl ab.** (ng/µl)	**Total ng**		**Input** (ng/µl)	**Total ng**		**IgG** (ng/µl)
1 million cells	0.15	6.1		0.49	19.7		9.81	392.35		0.1
0.5 million cells	0.12	4.71		0.1	4.15		3.38	135.2		
0.25 million cells	0.1	4.04		0.11	4.45		1.7	67.93		
MOUSE	**4µl ab.** (ng/µl)	**Total ng**		**2µl ab.** (ng/µl)	**Total ng**		**Input** (ng/µl)	**Total ng**		**IgG** (ng/µl)
1 million cells	0.1	3.95		0.1	4.01		5.33	213.36		0.1
0.5 million cells	0.1	3.91		0.1	4.17		2.37	94.66		
0.25 million cells	0.1	3.92		0.1	3.92		0.9	35.86		

Quantitative PCR

Positive target primers: ACTB-2 (2)
Negative target primers: ZNF333 (6)

HUMAN

		Positive	**Average IP CT**	**% of input**	**Negative**	**Average IP CT**	**% of input**
4µl	*1x10e6 cells*	27.18	27.03	5.54	34.70	34.48	0.09
4µl		26.88			34.26		
4µl	*.5x10e6 cells*	34.24	33.57	0.91	35.73	35.03	0.37
4µl		32.90			34.33		
4µl	*.25x10e6 cells*	31.12	30.83	34.15	32.63	32.82	10.51
4µl		30.54			33.00		
		Positive	**Average IP CT**	**% of input**	**Negative**	**Average IP CT**	**% of input**
2µl	*1x10e6 cells*	28.91	28.75	1.69	34.02	34.23	0.11
2µl		28.58			34.44		
2µl	*.5x10e6 cells*	33.44	33.08	1.27	33.68	33.80	0.88
2µl		32.72			33.91		
2µl	*.25x10e6 cells*	36.08	35.01	1.88	36.68	36.68	0.72
2µl		33.94					
		Positive	**Average IP CT**	**% of input**	**Negative**	**Average IP CT**	**% of input**
2ug	*IgG 1x10e6*	36.31	38.55	0.00		36.52	0.02
2ug		40.79			36.52		
		Positive	**Adjusted CT**	**Avg. Input**	**Negative**	**Adjusted CT**	**Avg. Input**
	Input 1x10e6	26.56	23.24	22.86	27.75	24.43	24.43
		25.79	22.47		27.74	24.42	
	Input 0.5x10e6	30.46	27.14	26.79	30.30	26.98	26.96
		29.75	26.43		30.26	26.94	
	Input 0.25x10e6	32.88	29.56	29.28	32.78	29.46	29.57
		32.32	29.00		32.99	29.67	

Date: 03/06/14 Experiment #: ID.1.14.27
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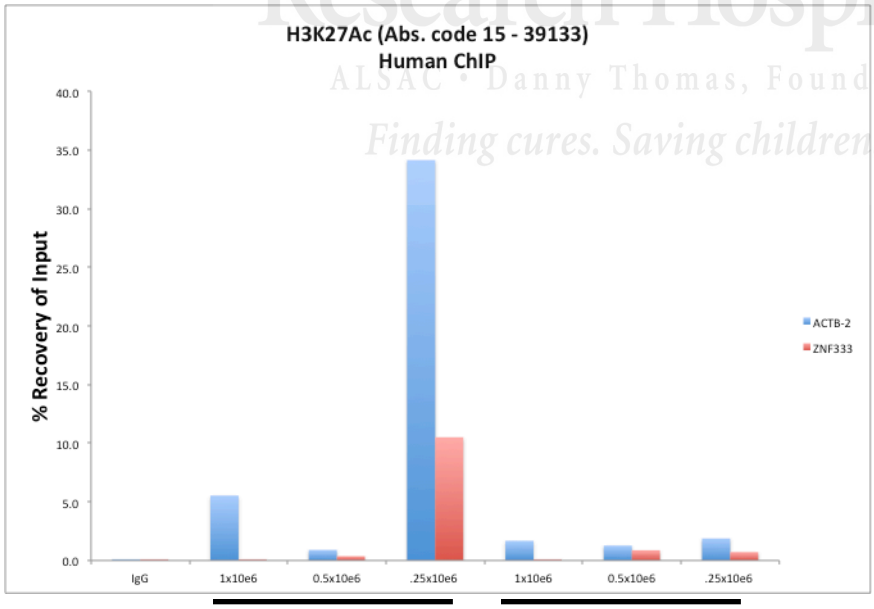
Positive target primers: Gapdh (18)
Negative target primers: GD Chr6 (13)

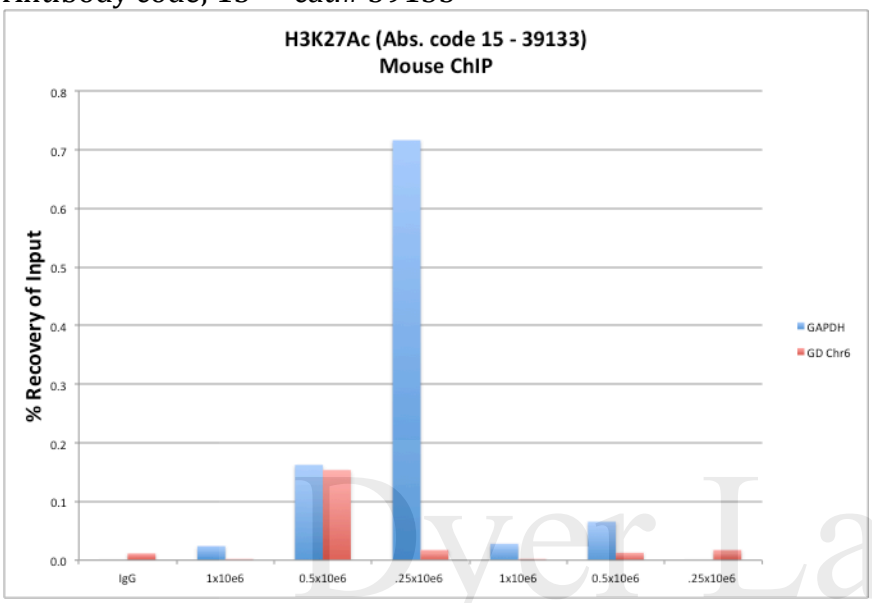
MOUSE

		Positive	Average IP CT	% of input	Negative	Average IP CT	% of input
4µl	*1x10e6 cells*	35.01	35.09	0.02	40.79	38.98	0.00
4µl		35.16			37.16		
4µl	*.5x10e6 cells*	34.78	35.38	0.16	34.97	35.08	0.15
4µl		35.97			35.18		
4µl	*.25x10e6 cells*	35.93	35.93	0.72	40.79	40.79	0.02
4µl		35.01	35.09	0.02	40.79	38.98	0.00
		Positive	**Average IP CT**	**% of input**	**Negative**	**Average IP CT**	**% of input**
2µl	*1x10e6 cells*	34.87	34.87	0.03	36.60	38.70	0.00
2µl					40.79		
2µl	*.5x10e6 cells*	36.68	36.68	0.07	40.79	38.66	0.01
2µl					36.53		
2µl	*.25x10e6 cells*		#DIV/0!	#DIV/0!	40.79	40.79	0.02
2µl					40.79		
		Positive	**Average IP CT**	**% of input**	**Negative**	**Average IP CT**	**% of input**
2ug	*IgG 1x10e6*		#DIV/0!	#DIV/0!	35.72	35.72	0.01
2ug					35.08		
		Positive	**Adjusted CT**	**Avg. Input**	**Negative**	**Adjusted CT**	**Avg. Input**
	Input 1x10e6	26.48	23.16	23.07	25.94	22.62	22.65
		26.30	22.98		25.99	22.67	
	Input 0.5x10e6	29.37	26.05	26.11	28.95	25.63	25.73
		29.49	26.17		29.15	25.83	
	Input 0.25x10e6	32.36	29.04	28.81	31.85	28.53	28.32
		31.89	28.57		31.42	28.10	



4µl abs. 2µl abs.

Date: 03/06/14 Experiment #: ID.1.14.27
Antibody code; 15 cat.# 39133



Conclusions: (provide yes or no answer)

Human Tissue
The ratio of the positive versus negative control targets is 5 or higher: YES

Antibody passes validation for level 2: YES
I recommend 4µl of antibody per 1x10e6 human cells

Mouse Tissue
The ratio of the positive versus negative control targets is 5 or higher: YES- borderline pass

This is a borderline pass because the CT values are very high. This can possibly improve with higher yield of recovered DNA.

Antibody passes validation for level 2: YES

I recommend 4µl of antibody per 1x10e6 mouse cells.

Additional notes:

This antibody is recommended for use in ChIP-seq experiments. I also recommend using 2-3 positive and negative target genes for qPCR.

Based on DNA quantification data for the mouse retina, I would recommend scaling up the ChIP reaction by at least 2-4 times the original volume in order to get a DNA yield that is measurable. At least 15ng is required for submission to PCGP for ChIP-seq library prep.