

SUPPLEMENTARY INFORMATION: DATA VALIDATION

Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project.

This supplement provides the links to the key validation datasets, published separately for each class of experiments in the ENCODE project. In each case, a brief introduction of the data type is provided and then a DOI (Digital Object Identifier) and a URL which will resolve to the correct article. Where appropriate we have provided a short guide to which key figures or tables in the paper are used for validation. Most of the papers are found in the ENCODE special issue of Genome Research.

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1. Quantitative real-time PCR (qRT-PCR) validation of ChIP-chip data from the NimbleGen platform (Ren Lab)

Validation of the Chip/chip data for the CTCF data was provided by qRT-PCR as described in the following paper:

Tae Hoon Kim, Ziedulla K. Abdullaev, Andrew D. Smith, Keith A. Ching, Dmitri I. Loukinov, Roland D. Green, Michael Q. Zhang, Victor V. Lobanenkov, and Bing Ren. 2007. Analysis of the Vertebrate Insulator Protein CTCF-Binding Sites in the Human Genome. *Cell* **128**: 1231-12 doi:10.1016/j.cell.2006.12.048.

DOI: doi:10.1016/j.cell.2006.12.048

URL: <http://dx.doi.org/doi:10.1016/j.cell.2006.12.048>

From the CTCF binding sites identified in the human genome, 84 sites were randomly selected for conventional ChIP analysis. For negative controls, 17 promoters sequences that were not near any CTCF binding sites were selected. Quantitative real-time PCR was performed with 0.5 ng of CTCF ChIP DNA and unenriched total genomic DNA. The quantitative real-time PCR of each sample was performed in duplicate using iCycler™ and SYBR green iQ™ SYBR green supermix reagent (Bio-Rad Laboratories). The threshold cycle (Ct) values were calculated automatically by the iCycle iQ™ Real-Time Detection System Software (Bio-Rad Laboratories). Normalized Ct (ΔCt) values for each sample were then calculated by subtracting the Ct value obtained for the unenriched DNA from the Ct value for the CTCF ChIP DNA ($\Delta Ct = Ct_{ctcf} - Ct_{total}$). The fold enrichment of the tested promoter sequence in ChIP DNA over the unenriched DNA was then estimated. Primers used for this analysis and the data are found in <http://dx.doi.org/doi:10.1016/j.cell.2006.12.048> in Supplement Table S1, S2 and S3.

The Validation of the histone modification, PolIII, TAFI has not been published separately

To validate the ChIP/chip data for H3K4me2, H3K4me3, H3ac, H4ac, RNA PolIII, TAF1, Chromatin from four biological replicates of HeLa S3 cells was isolated and immunoprecipitated using appropriated antibodies. ChIP DNA was purified, replicate samples were pooled and quantified, and 0.5 ng of ChIP DNA and corresponding input DNA were assayed in duplicate by quantitative real-time PCR using iCycler™ and SYBR-green iQ™ Supermix (Bio-Rad Laboratories, 170-8882). The cycle-number differences between ChIP DNA and input DNA were normalized using a pool of random controls, and were scored as positive if the differences were 2 standard deviations above the average of the controls. Three sets of genomic regions were selected and tested:

Group I: A. Between 11-13 of these were from the False Discovery Rate of 1% (FDR1); B. Between 6-12 of these were selected from fdr5, but did not overlap with fdr1; C. Between 2-13 of these were selected from fdr10, but did not overlap with fdr1 nor fdr5 lists; D. Between 1-4 of these were selected from results from the PCR product arrays, but did not overlap with the fdr1, fdr5, and fdr10 lists.

The validation rate for each category was computed as # of positives/ total tested.

Group II. To estimate the specificity of the results, 48 random genomic regions from the ENCODE sequences were selected, primers were designed and real-time quantitative PCR was performed to test their enrichment in ChIP DNA, compared to the input DNA. The number of negatives in this assay was compared to that of the ChIP-chip hit lists at different cutoffs. If the region is negative by qPCR but positive by ChIP-chip, then it is counted as a false positive (FP). If the region is negative by both methods then it is counted as true negatives (TN). The specificity is calculated as $TN/(TN + FP)$.

Group III. To estimate the sensitivity of the results, 48 known promoters were selected. Primers were designed and real-time quantitative PCR was performed to test their enrichment in ChIP DNA, compared to input DNA. The number of positives in this assay was compared to that of the ChIP-chip hit lists at different cutoffs. If the region is positive by qPCR but negative by ChIP-chip, then it is counted as a false negative (FN). If the region is tested positive by both methods, then it is counted as a true positive (TP). The sensitivity is calculated as $TP/(TP + FN)$.

The results of the validation experiments are listed below:

	H3K4me2	H3K4me3	H3ac	H4ac	RNA PolII	TAF1
Random Controls						
1% FDR	45/45 100.00	46/46 100.00	44/44 100.00	45/47 95.74	47/47 100.00	46/47 97.87
5% FDR	45/45 100.00	46/46 100.00	44/45 97.78	45/47 95.74	47/47 100.00	46/47 97.87
10% FDR	45/45 100.00	46/46 100.00	44/46 95.65	45/47 95.74	47/48 97.92	46/47 97.87
Positive Controls						
1% FDR	28/30 93.33	30/31 96.77	30/33 90.91	13/26 50.00	18/30 60.00	16/33 48.48
5% FDR	30/30 100.00	31/31 100.00	33/33 100.00	18/26 69.23	22/30 73.33	20/33 60.61
10% FDR	30/30 100.00	31/31 100.00	33/33 100.00	20/26 76.92	25/30 83.33	22/33 66.67
PCR Arrays Only	30/30 100.00	31/31 100.00	33/33 100.00	76.92	25/30 83.33	22/33 66.67
Validation						
1% FDR	10/12 83.33	10/11 90.91	8/11 72.73	9/13 69.23	12/12 100.00	13/13 100.00
5% FDR	3/12 25.00	1/12 8.33	5/6 83.33	9/11 81.82	12/12 100.00	10/11 90.91
10% FDR	2/13	2/12	0/2	8/11	5/6	10/11

	15.38	16.67	0.00	72.73	83.33	90.91
PCR Arrays	1/4	2/2	1/3	1/4	1/1	1/3
only	25.00	100.00	33.33	25.00	100.00	33.33

2. Validation of histone modification ChIP-chip data by qPCR (Dunham Lab)

Koch, C.M., Andrews, R.M., Flicek, P., Dillon, S.C., Karaöz, U., Clelland, G.K., Wilcox, S., Beare, D.M., Fowler, J.C., Couttet, P., et al. 2007. The landscape of histone modifications across 1% of the human genome in five human cell lines. *Genome Res.* doi: 10.1101/gr.5704207

DOI: 10.1101/gr.5704207

URL: <http://dx.doi.org/10.1101/gr.5704207>

In order to validate the ChIP-CHIP data, we identified and developed working assays for 74 enriched regions and 27 background signal regions for testing by quantitative PCR (qPCR) on anti-H3K4me3 ChIP material from the GM06990 cell line (for results, see Koch et al. doi:10.1101/gr.5704207).² By designing qPCR assays tiling through six representative ChIP-CHIP enrichment peaks we were able to reproduce qualitatively the enrichment profiles observed on the microarray (see Figure S2, Koch et al. doi:10.1101/gr.5704207).

Real time PCR

The enrichment (relative copy number) was determined in real-time PCR reactions using either ChIP DNA or non enriched DNA as template. For primer pair design and methods see Koch et al. doi:10.1101/gr.5704207).

3. Validation of Replication Timing Data (Dutta Lab)

Karnani, N., Taylor, C., Malhotra, A., Dutta, A. 2007. Pan-S replication patterns and chromosomal domains defined by genome tiling arrays of ENCODE genomic areas. *Genome Res.* doi: 10.1101/gr.5427007.

DOI: 10.1101/gr.5427007

URL <http://dx.doi.org/10.1101/gr.5427007>

The tiling array based replication timing data from HeLa cells has been validated by interphase FISH and is detailed in Karnani et al. doi:10.1101/gr.5427007. Interphase FISH probes from ENCODE regions are listed alongside FISH results of replication time in HeLa cells released from thymidine/aphidicolin block in Karnani et al. Supplemental Table 2.

Tiling array data for time of replication and interphase FISH results for validating replication timing in HeLa are presented in Karnani et al. Figures 2 and 3, respectively.

4. Verification of HL60 cytosolic polyA+ RNA TxFrag results (Affymetrix Group - Phil Kapranov)

Denoeud, F., Kapranov, P., Ucla, C., Frankish, A., Castelo, R., Drenkow, J., Lagarde, J., Alioto, T., Manzano, C., Chrast, J. 2007. Prominent use of distal 5' transcription start sites and discovery of a large number of additional exons in ENCODE regions. *Genome Res.* doi: 10.1101/gr.5660607.

This section is taken from the Supplementary Information of the present manuscript: The ENCODE Consortium. The ENCODE pilot project: Identification and analysis of functional elements in 1% of the human genome and from Denoeud et al. doi:10.1101/gr.5660607.

DOL: 10.1101/gr.5660607

URL: <http://dx.doi.org/10.1101/gr.5660607>

Validation Supplement Table 2 Validation results of Affymetrix genome tiling array maps

	Index TF	Successful RACE reactions (%)				Transcription on both strands	No transcript detected
		5' RACE	3' RACE	5' and 3' RACE	5' or 3' RACE		
Exonic	20	19 (95)	19 (95)	16 (80)	20 (100)	19 (95)	0 (0)
Intronic	90	71 (79)	77 (86)	66 (73)	79 (88)	65 (72)	11 (12)
Intergenic	90	62 (69)	65 (72)	44 (49)	77 (86)	46(51)	13 (14)
Non Transfrag Regions	100	66 (66)	60 (60)	45 (45)	75 (75)	44 (44)	25 (25)

Numbers represent transfrags. Numbers in () represent % of total number of regions tested.

200 transfrags were randomly chosen from the map of HL60 cell line un-stimulated (00hr time point) with retinoic acid. The transfrags consisted of 90 intergenic transfrags, 90 intronic and 20 exonic transfrags. Intergenic or intronic transfrags were defined as correspondingly non-overlapping or overlapping the bounds of known genes from the UCSC Known Gene track on the hs.NCBIv35 version of the genome. Intergenic and intronic transfrags were selected not to overlap any mRNA or EST annotation. Information on the index transfrags, primers used for this analysis can be found at <http://genome.imim.es/gencode/RACEdb>. 100 non-transfrag regions that mimic transfrags in length were randomly selected throughout the non-repetitive portions of the ENCODE regions.

5' and 3' RACE analysis was performed on DNaseI-treated cytosolic polyA+ RNA from un-stimulated HL60 cell line for each transfrag for each strand of the genome totaling to 4 RACE reactions per transfrag. RACE reactions were performed essentially as described in Kapranov et al⁶ with the following modifications. cDNA synthesis for the 5'RACE was performed with a pool of 12 gene-specific primers. cDNA synthesis was done with two reverse-transcriptases: Superscript II and Thermoscript (both form

Invitrogen) in two separated reactions with 50 ng of polyA+ RNA each. The cDNA reactions were pooled for the RT-PCR step. cDNA synthesis for the 3'RACE was performed with oligo-dT 3' CDS primer as in Kapranov et al⁵. The cDNA was treated with RNase A/T1 cocktail (Ambion) and RNase H (Epicentre), purified over Qiagen's columns and pooled for the RT-PCR step. 40 ng of purified cDNA were used as starting material for each RT-PCR reaction. Three rounds of amplifications were performed at the RT-PCR step of the RACE utilizing 3 transfrag-specific nested RT-PCR primers for both 3' and 5' RACE. After each round, the RT-PCR reactions were purified using QIAquick 96 PCR purification system (Qiagen) and eluted in 70 µl. 1 µl of the first round amplification was used as a template for the second round and 0.01 µl of the second round RT-PCR reaction was used as a template for the third round. Oligonucleotides 3' CDS, UPL/UPS and NUP (Clontech SMART II RACE protocol) were used as common primers for the first, second and third round of RT-PCR. Each round of amplification consisted of 25 cycles of PCR (94°C for 20 sec; 62°C for 30 sec; 72°C for 5 min) followed by 10 min at 72°C. Products of the final round of RT-PCRs were purified using QIAquick 96, pooled and hybridized to ENCODE arrays as described above. The maps were generated using the Tiling Analysis Software (TAS; <http://www.affymetrix.com/support/developer/downloads/TilingArrayTools/index.affx>) with bandwidth of 50. RACEfrags were generated using threshold of 100, maxgap =50 and minrun =50.

The Affymetrix RACEfrags were filtered so that each pool contains RACEfrags that are unique to the pool. GENCODE RACEfrags were filtered against Affymetrix RACEfrags. Regions overlapping RACEfrags from the Affymetrix pools were removed. Pooling was done so that the index transfrags within each pool are at least 40 kbp apart from each other. This is to facilitate the unambiguous assignment of the parent child relationships between the index transfrag and the RACEfrag. A region (transfrag or non-transfrag) was considered to be positive for presence of a transcript of either 5' or 3' RACE reaction was scored positive on either strand.

To control for genomic DNA contamination, 3' RACE reactions were conducted on the 100 non-transfrag regions with the omission of the reverse transcriptase. Only 1 region was scored as positive.

The data for the entire verification dataset can be loaded from a centralized RACE database RACEdb located at this URL <http://genome.imim.es/gencode/RACEdb>. Also, the profile of each RACE reaction for each of the 300 index regions could be viewed via the links provided in this database in the UCSC browser or loaded as a BED file.

5. Validation experiments for DNase-chip (Crawford Group)

Crawford, G. E. et al. DNase-chip: a high-resolution method to identify DNase I hypersensitive sites using tiled microarrays. *Nat Methods* 3, 503-9 (2006)

DOI: doi:10.1038/nmeth888

URL: <http://dx.doi.org/doi:10.1038/nmeth888>

Validation was provided by Real time PCR on putative DNaseHS sites. 7 different categories of sites were used depending on the presence of sites at different DNase concentrations. Data is shown in Figure 2a,b and in Supplementary Table 1.

6. Experimental Testing of Connectivity of Genomic Regions (Yale Group – Joel Rozowsky)

Rozowsky, J., Newburger, D., Sayward, F., Wu, J., Jordan, G., Korbel, J.O., Nagalakshmi, U., Yang, J., Zheng, D., Guigo, R., et al. 2007. The DART classification of unannotated transcription within the ENCODE regions: Associating transcription with known and novel loci. *Genome Res.* doi: 10.1101/gr.5696007.

DOI: 10.1101/gr.5696007

URL: <http://dx.doi.org/10.1101/gr.5696007>

RT-PCR and Sequencing was carried out for novel TARs expressed in placental RNA as described in Rozowsky et al. doi:10.1101/gr.5696007.¹⁰ A table of the regions selected for RT-PCR validation testing for connectivity and their corresponding annotation and primer sequences are indicated in Rozowsky et al. Supplemental Table 1.

The sequences, forward and reverse primer sequences, the length of the unspliced genomic span between the primers and the length of the sequenced product obtained for PCR products that were sequenced is displayed in Rozowsky et al. Supplemental Table 2.

7. Validation of STAT1 ChIP-chip and ChIP-PET (Yale Group – Mike Snyder)

Euskirchen, G.M., Rozowsky, J., Wei, C.-L., Lee, W.H., Zhang, Z.D., Hartmen, S., Emanuelsson, O., Stolc, V., Weissman, S., Gerstein, M., et al. 2007. Mapping of transcription factor binding regions in mammalian cells by ChIP: Comparison of array and sequencing based technologies. *Genome Res.* doi: 10.1101/gr.5583007

DOI: 10.1101/gr.5583007

URL: <http://dx.doi.org/10.1101/gr.5583007>

Yale Validation

Stat1 ChIP-chip and ChIP-PET datasets were validated in at least two biological replicate experiments using a quantitative PCR assay. Details of this analysis are available in Euskirchen et al. doi:10.1101/gr.5583007¹¹.

8. Validation of DnaseI hypersensitive site (DHS) mapping by DNase-array and QCP (Stamatoyannopoulos Lab)

Quantitative Chromatin Profiling (QCP)

Dorschner MO, Hawrylycz M, Humbert R, Wallace JC, Shafer A, Kawamoto J, Mack J, Hall R, Goldy J, Sabo PJ, Kohli A, Li Q, McArthur M, Stamatoyannopoulos JA. High-throughput localization of functional elements by quantitative chromatin profiling. *Nat Methods* 1, 219-25 (2004).

DOI: 10.1038/nmeth721

URL <http://dx.doi.org/10.1038/nmeth721>

To validate that DHSs identified by QCP correspond with classical DnaseI hypersensitive sites, we compared QCP data (high-throughput real-time quantitative PCR using end-to-end tiled ~225bp amplicons) with previously published DNaseI hypersensitivity Southern data from multiple loci (alpha and beta-globin, T-cell receptor alpha, c-myc, adenosine deaminase, CD2) in erythroid, lymphoid, and hepatic cell types. 100% of known DHSs were detected. Novel sites were verified by conventional Southern blotting. Results are shown in Figs. 2-6, Supplementary Figs. 1-2, and Supplementary Table 1.

In unpublished studies during the ENCODE Pilot Project we additionally performed extensive conventional DnaseI hypersensitivity mapping in GM06990, CACO2, K562, HepG2, and SKnSH chromatin using conventional hypersensitivity Southernblots. In total we performed 1,114 Southern end-label experiments. On the basis of these experiments the sensitivity of QCP was calculated to be 94.1% and specificity 99.8%.

Dnase-array

Sabo PJ, Kuehn MS, Thurman R, Johnson BE, Johnson EM, Cao H, Yu M, Rosenzweig E, Goldy J, Haydock A, Weaver M, Shafer A, Lee K, Neri F, Humbert R, Singer MA, Richmond TA, Dorschner MO, McArthur M, Hawrylycz M, Green RD, Navas PA, Noble WS, Stamatoyannopoulos JA. Genome-scale mapping of DNase I sensitivity in vivo using tiling DNA microarrays. *Nat Methods* 3, 511-8 (2006)

DOI: 10.1038/nmeth890

URL <http://dx.doi.org/10.1038/nmeth890>

To validate that DHSs identified by DNase-array correspond with classical DnaseI hypersensitive sites, we performed extensive conventional DnaseI hypersensitivity Southern mapping in lymphoblastoid (GM06990) cells. Results are shown in Fig. 2, Supplementary Fig. 1, Table 1 and Supplementary Table 2.

9. Validation of E2F1 ChIP-chip results (Farnham Lab)

Mark Bieda, Xiaoqin Xu, Michael A. Singer, Roland Green and Peggy J. Farnham Unbiased location analysis of E2F1-binding sites suggests a widespread role for E2F1 in the human genome

DOI : 10.1101/gr.4887606

URL: <http://dx.doi.org/10.1101/gr.4887606>

PCR quantification was performed using standard methods; in brief, primers were designed to test a subset of predicted binding sites. The PCR product for the ChIP sample was compared to the control sample. PCR confirmation experiments are described in Bieda et al. doi:10.1101/gr.4887606 Results of PCR confirmation of peak predictions are in Bieda et al. Supplemental Table 1.

10. Quantitative PCR (qPCR) validation of FAIRE sample (Iyer and Lieb Labs)

Paul G. Giresi, Jonghwan Kim, Ryan M. McDaniel, Vishwanath R. Iyer, and Jason D. Lieb. 2006. FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. *Genome Res.* Published December 19, doi:10.1101/gr.5533506.

DOI: 10.1101/gr.5533506

URL: <http://dx.doi.org/10.1101/gr.5533506>

The FAIRE procedure and qPCR validation is described in Giresi et al. 10.1101/gr.5533507.¹⁴ FAIRE validation results for a portion of chromosome 21 are displayed in Giresi et al. Figure 3.

11. Quantitative PCR validation of ChIP-chip data (Struhl Lab)

The validation of this data has not yet been separately published.

The validation qPCR was performed a previously described in Beida et al, *Genome Research*, 16, 595-605. using an Applied Biosystems 7000 sequence detector and an Applied Biosystems 7700 sequence detector for SYBR green fluorescence. The PCR program was: 95 oC 10 min, followed by 40 cycles of 95 oC, 30 sec, 60 oC, 45 sec; 72 oC, 1 min. Fold enrichment for a for a called site from an array region was determined relative to a non-enriched region reference region determined for each factor. The formula used was: fold enrichment = $1.9^{-(\Delta CT_{\text{expt}} - \Delta CT_{\text{ref}})}$ where ΔCT is the cycle threshold (Ct) difference between ChIP DNA and input material, calculated for experimental and reference regions, and 1.9 is the mean primer slope. Sites tested were selected to encompass a large range of pValues as well as several array regions that were not called sites for a specific factor for use as negative controls. A called site was considered a true site if the qPCR fold enrichment value was greater than or equal to 3 standard deviations above the mean of the negative controls sites.

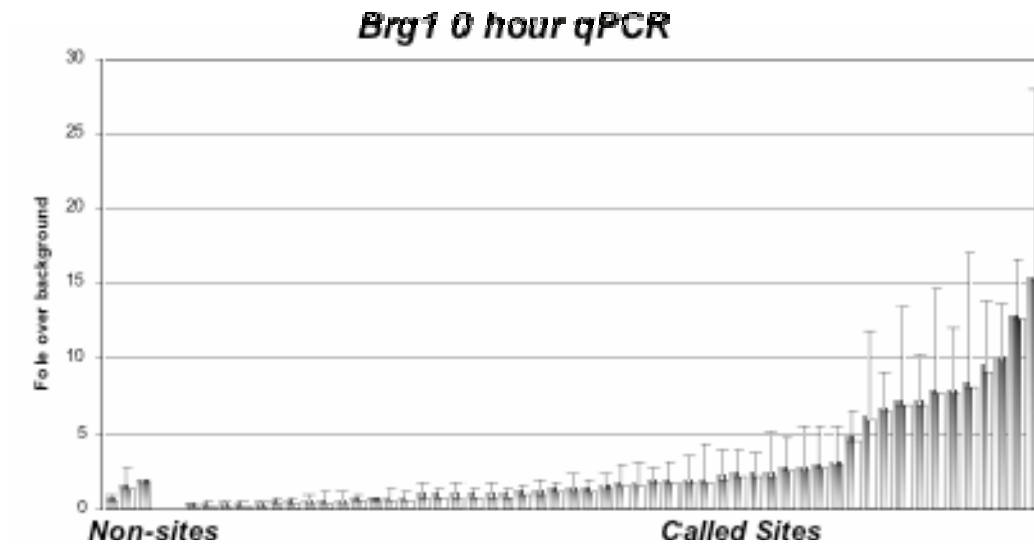
The pValue used to threshold the list of potential sites was determined by extrapolation qPCR results of true versus false sites along the list of pValues such that a 95% accuracy rate was required for qPCR. Therefore, by definition the final number of sites provided in table 1 are based on a 5% FDR. False negative rate was determined by calculating the percentage of true sites tested by qPCR that fall below

the pValue cutoff. This percentage was then applied to the original site lists to estimate the number of potential false negative sites for each factor.

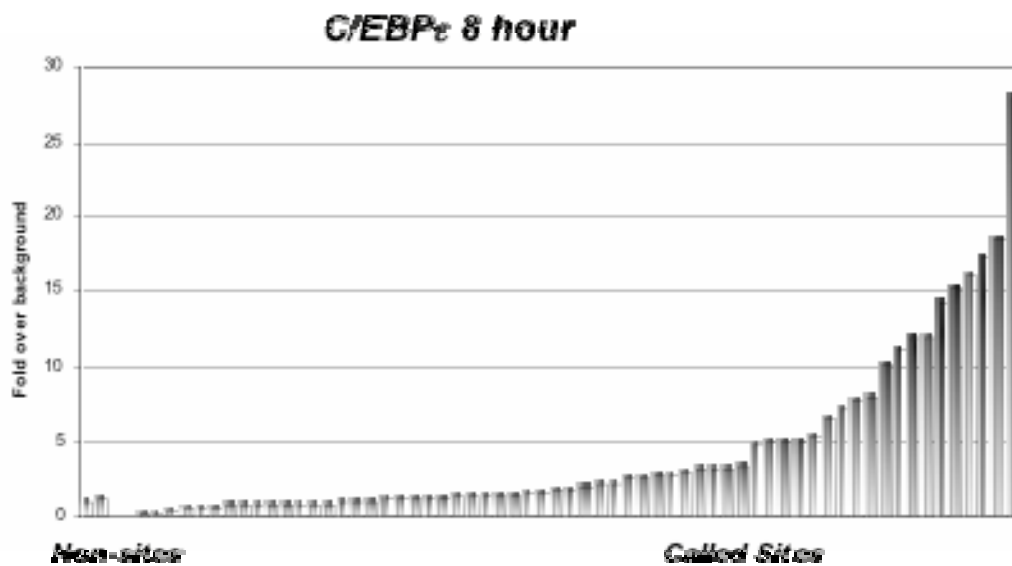
Validation Supplement Table 6: Summary of threshold sites, false discovery, and false negative rates.

Factor	Time Point	Original Number of Sites	Factor Threshold pFDR	Threshold Sites	# True Positive Sites	# False Positive Sites	False Negative Rate	# False Negative Sites
Brg1	0 hour	1154	74	27	26	1	9.09%	102
	2 hour	117		2	2	0		10
	8 hour	717		16	15	1		64
	32 hour	417		15	14	1		37
CEBPe	0 hour	1351	70	195	185	10	30.20%	349
	2 hour	1176		163	155	8		306
	8 hour	1521		234	222	12		389
	32 hour	535		187	178	9		105
H3 Ac **	0 hour	97	36	96	96	NA	NA	NA
	2 hour	99		99	99	NA		NA
	8 hour	117		116	116	NA		NA
	32 hour	102		100	100	NA		NA
H4 Ac	0 hour	2371	56	1101	1046	55	43.30%	546
	2 hour	891		508	483	25		165
	8 hour	1914		727	691	36		513
	32 hour	1149		671	637	34		207
H3K27 Tri-ME	0 hour	1672	50	213	202	11	64.50%	941
	2 hour	1525		285	271	14		800
	8 hour	2769		513	487	26		1455
	32 hour	1439		233	221	12		778
Pol2	0 hour	1363	76	310	295	16	35.50%	374
	2 hour	479		155	147	8		115
	8 hour	819		242	230	12		205
	32 hour	1149		396	376	20		267
p300	0 hour	1251	95	11	10	1	50.00%	620
	2 hour	2852		65	62	3		1394
	8 hour	2506		60	57	3		1223
	32 hour	910		22	21	1		444
Skp1	0 hour	237	60	2	2	0	21.43%	50
	2 hour	557		4	4	0		118
	8 hour	219		2	2	0		46
	32 hour	505		11	10	1		106

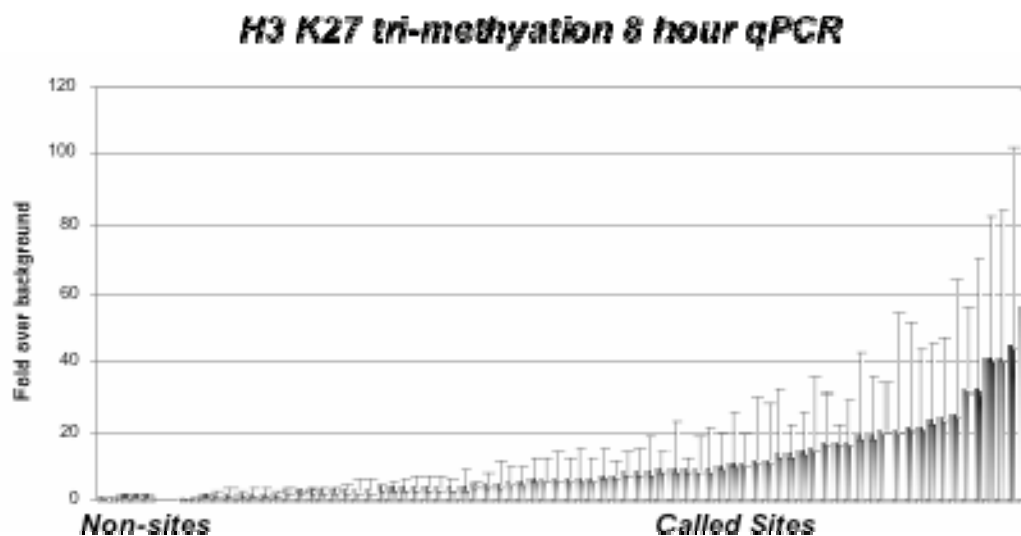
**Pvalue Threshold of H3 is 100% as all predicted sites tested by qPCR were positive and thus a 0% FDR is used for this factor.



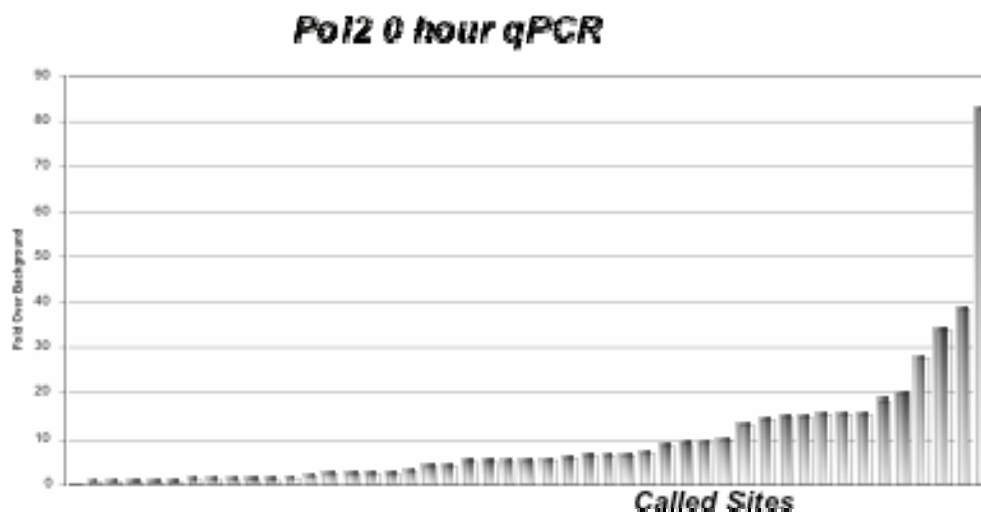
Validation Supplement Figure 1: QPCR validation of Brg1. 0 hour. 55 array regions were checked, including 52 called sites and 3 non-sites. The qPCR enrichment cut off is 3.17 fold.



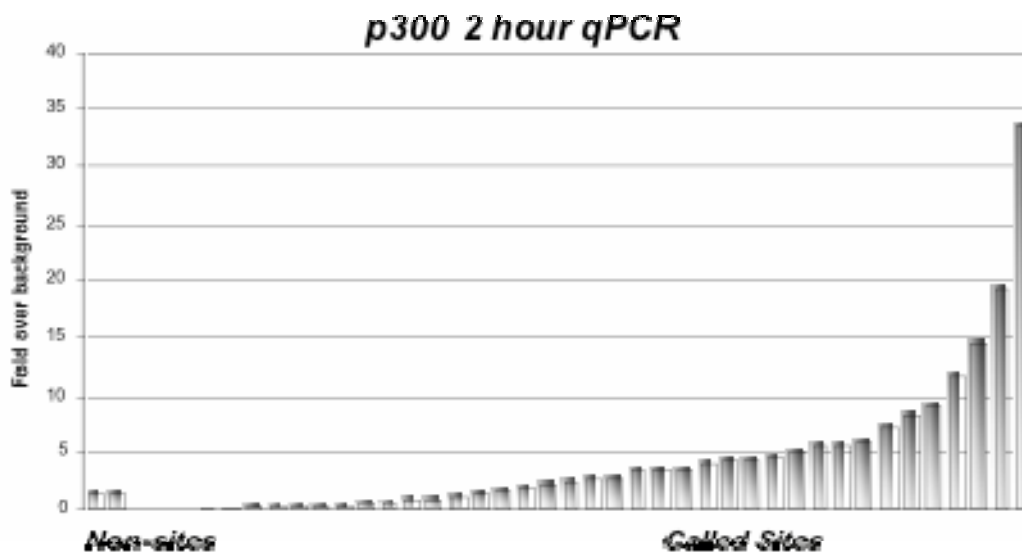
Validation Supplement Figure 2: QPCR validation of C/EBP ϵ . 8 hour. 64 array regions were checked, including 62 called sites and 2 non-sites. The qPCR enrichment cut off is 2 fold.



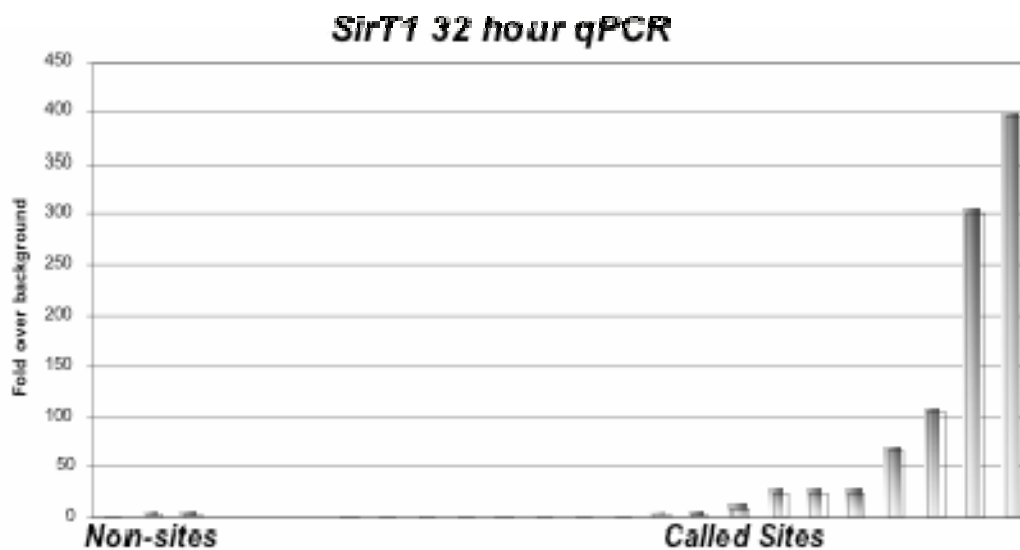
Validation Supplement Figure 5: QPCR validation of H3K27me3. 8 hour. 78 array regions were checked, including 73 called sites and 5 non-sites. The qPCR enrichment cut off is 2.34 fold.



Validation Supplement Figure 6: QPCR validation of RNA PolII. 0 hour. 52 array regions were checked including 47 called sites and 5 non-sites. The qPCR enrichment cut off is 3 fold.



Validation Supplement Figure 7: OPCR Validation of p300. 2 hour. 40 array regions were checked, including 38 called sites and 2 non-sites. The qPCR enrichment cut off is 2 fold.



Validation Supplement Figure 8: QPCR validation of SirT1. 32 hour. 22 array regions were checked, including 19 called sites and 3 non-sites. The qPCR cut off is 6.99 fold.