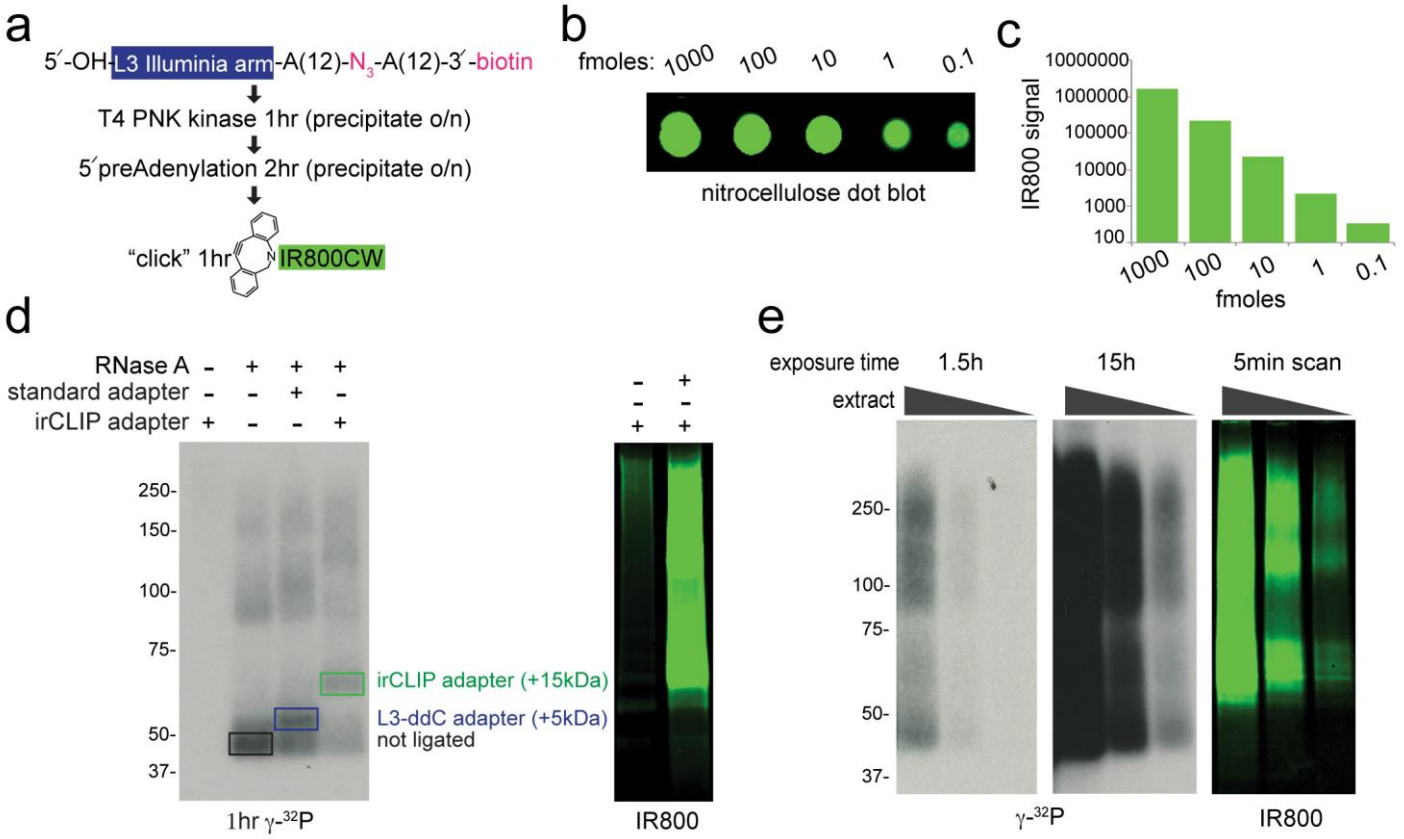
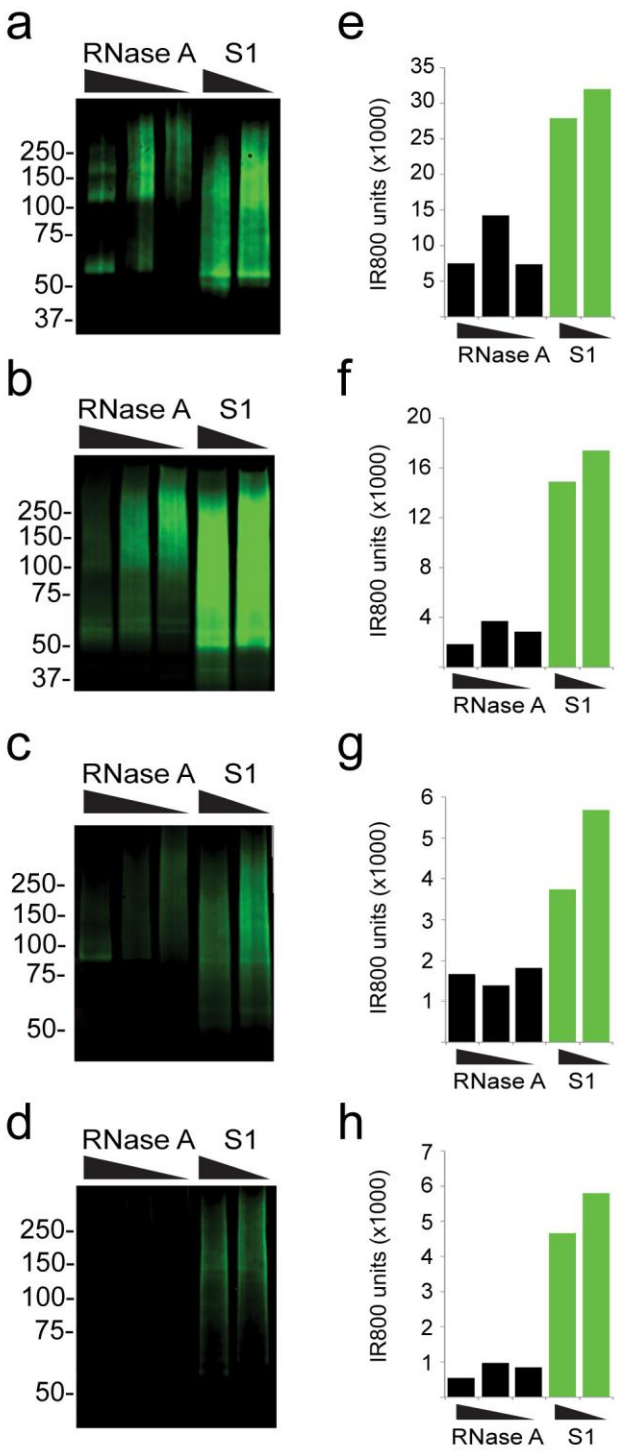


Supplementary Figure 1

irCLIP adapter construction, quantitation and validation

(**a**) Construction schematic for generation of the pre-adenylated, IR800CW dye conjugated irCLIP DNA adapter. (**b**) Odyssey CLx infrared scan and (**c**) quantitation of serial diluted irCLIP adapter showing 5 orders of magnitude sensitivity on nitrocellulose. (**d**) Autoradiogram and Odyssey CLx infrared scan demonstrating efficient ligation of γ–^{32}P-labeled hnRNP-C-RNA complexes with the irCLIP adapter, evidenced by supershifting of unligated complexes (black vs green box, left panel) and RNase A (in-lysate digestion, 50 ng/ml) dependence for generation of irCLIP adapter signal (right panel). (**e**) Autoradiograms (left and middle panels) and Odyssey CLx infrared scan (right panel) of a 3-fold serial dilution of γ–^{32}P -labeled, irCLIP-adapter ligated hnRNP-C-RNA complexes.

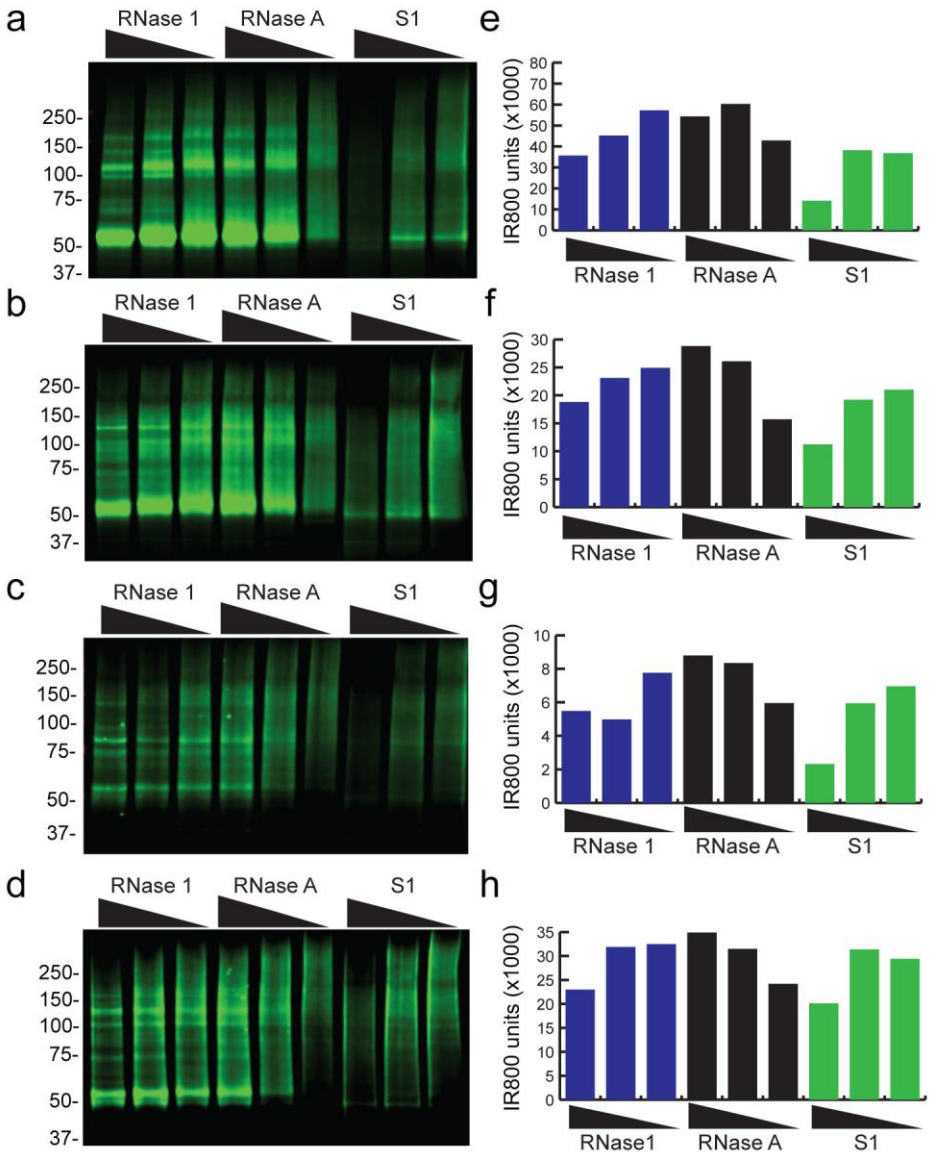


Supplementary Figure 2

On-bead S1 nuclease digestion improves RNA digestion profile for multiple classes of RBPs

Equivalent amounts of UV-C treated HeLa cell extracts were pre-digested with RNase A at 50, 16.7, or 5.6 ng/mL for 10 min at 37°C or digested post-immunoprecipitation with S1 nuclease at 9 or 3 U/μL for 15 min at

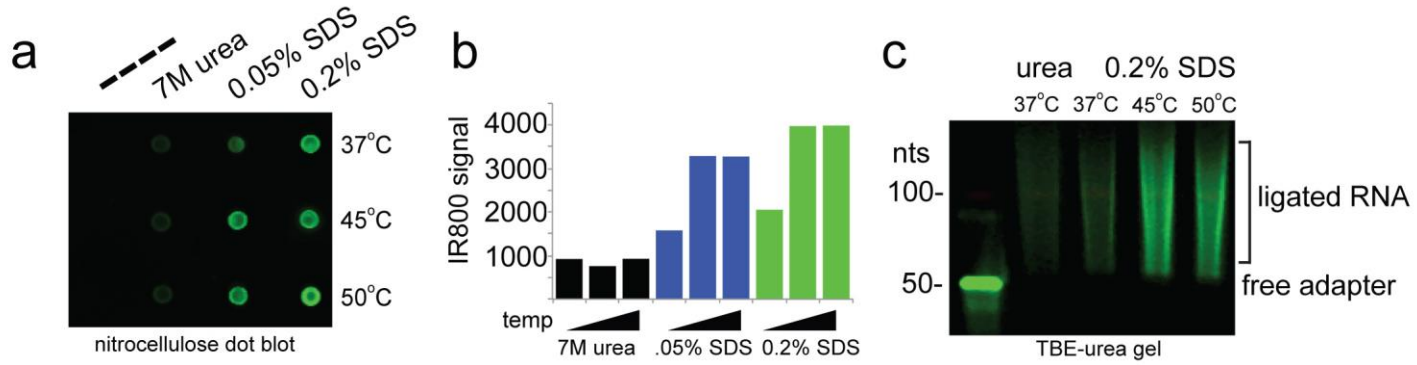
30°C, ligated overnight with the irCLIP-adapter, resolved by SDS-PAGE and transferred to nitrocellulose. (**a-d**) Odyssey CLx infrared scans of the nitrocellulose bound immunoprecipitates using antibodies against the RRM domain proteins (**a**) hnRNP-C, 37 kDa (**b**) HuR, 39 kDa, the RNA-helicase-domain protein DDX5, 73 kDa (**c**), and the double-stranded-RNA-binding domain protein ILF3, 90 and 110 kDa (**d**). (**e-h**) Quantitation of optimal ligations, 40-100 nucleotide RNA fragments (~30-60 kDa window above RBP size) for hnRNP-C, HuR, DDX5, and ILF3, respectively.



Supplementary Figure 3

Comparison of on-bead nuclease digestions for multiple classes of RBPs

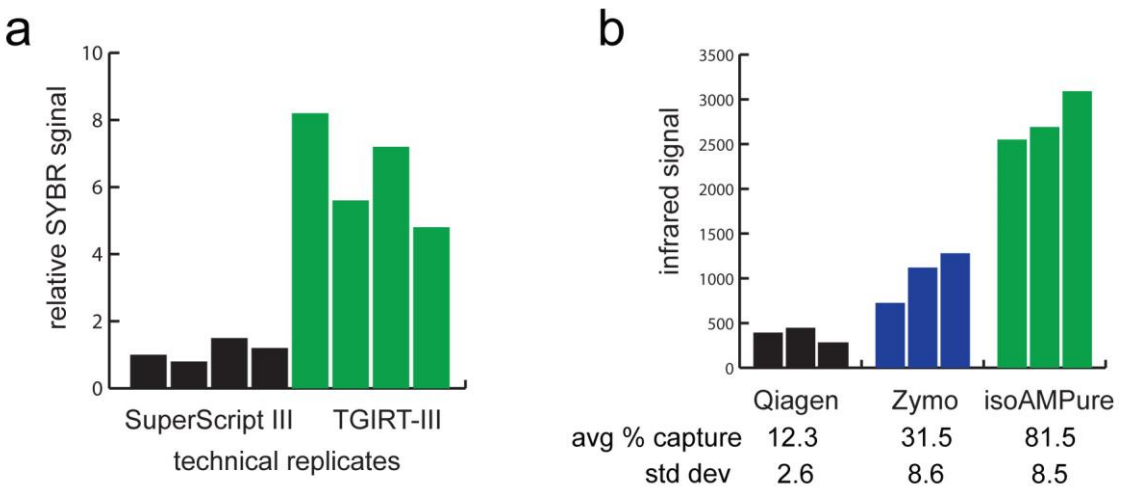
Batch immunoprecipitations were equally divided immediately prior to on-bead nuclease digestions. All reactions (see **Methods**) were incubated for 15 min at 30°C and each nuclease tested at 3 doses along a 4-fold serial dilution (RNase 1 0.4-0.1-0.025 U/μL, RNase A 20-5-1.25 ng/mL, S1 12-4-1 U/μL). Overnight irCLIP-adapter ligated RNP-complexes were resolved by SDS-PAGE. Odyssey CLx infrared scans of the nitrocellulose bound immunoprecipitates for (**a**) hnRNPC, 37 kDa, (**b**) HuR 39 kDa, (**c**) DDX5, 73 kDa and (**d**) ILF3 90 and 110 kDa. Quantitation of target ligations, 40-100 nucleotide RNA fragments (~30-60 kDa window above RBP size), (**e-h**) for hnRNP-C, HuR, DDX5, and ILF3, respectively.



Supplementary Figure 4

Optimized recovery of ligated RNA-peptide fragments

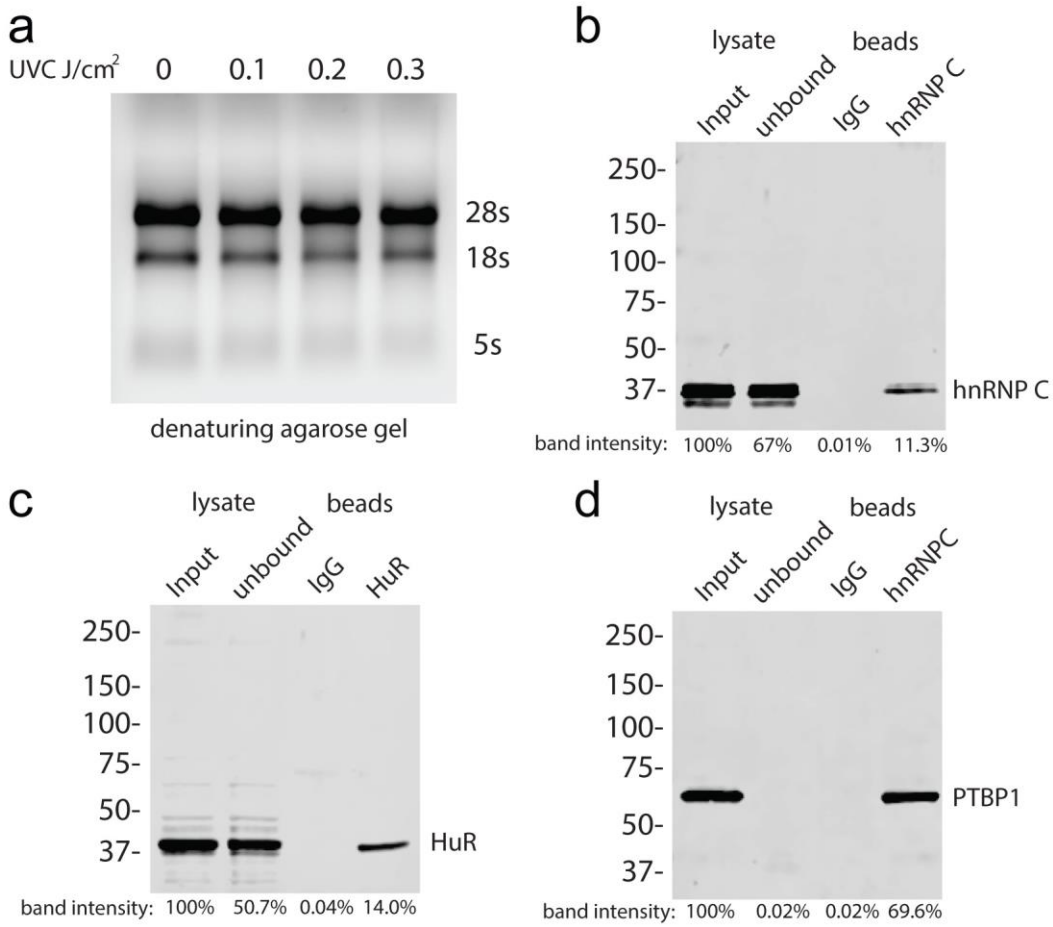
irCLIP-adapter ligated hnRNP-C-RNA complexes were diluted in 1xLDS sample buffer and equally divided between 36 lanes for resolution by SDS-PAGE. Identical sections, 65-100 kDa, of nitrocellulose were excised for comparison of proteinase K digestion parameters. (**a**) Infrared Odyssey CLx scan of representative nitrocellose dot blots and (**b**) quantitation of precipitated, irCLIP-adapter ligated RNA from proteinase K digestions with the indicated additives at the indicated temperatures. (**c**) Free irCLIP-adapter and representative RNA samples from (**a**) were resolved by denaturing PAGE and imaged on an infrared Odyssey CLx.



Supplementary Figure 5

irCLIP enhancement of cDNA synthesis and cDNA purification

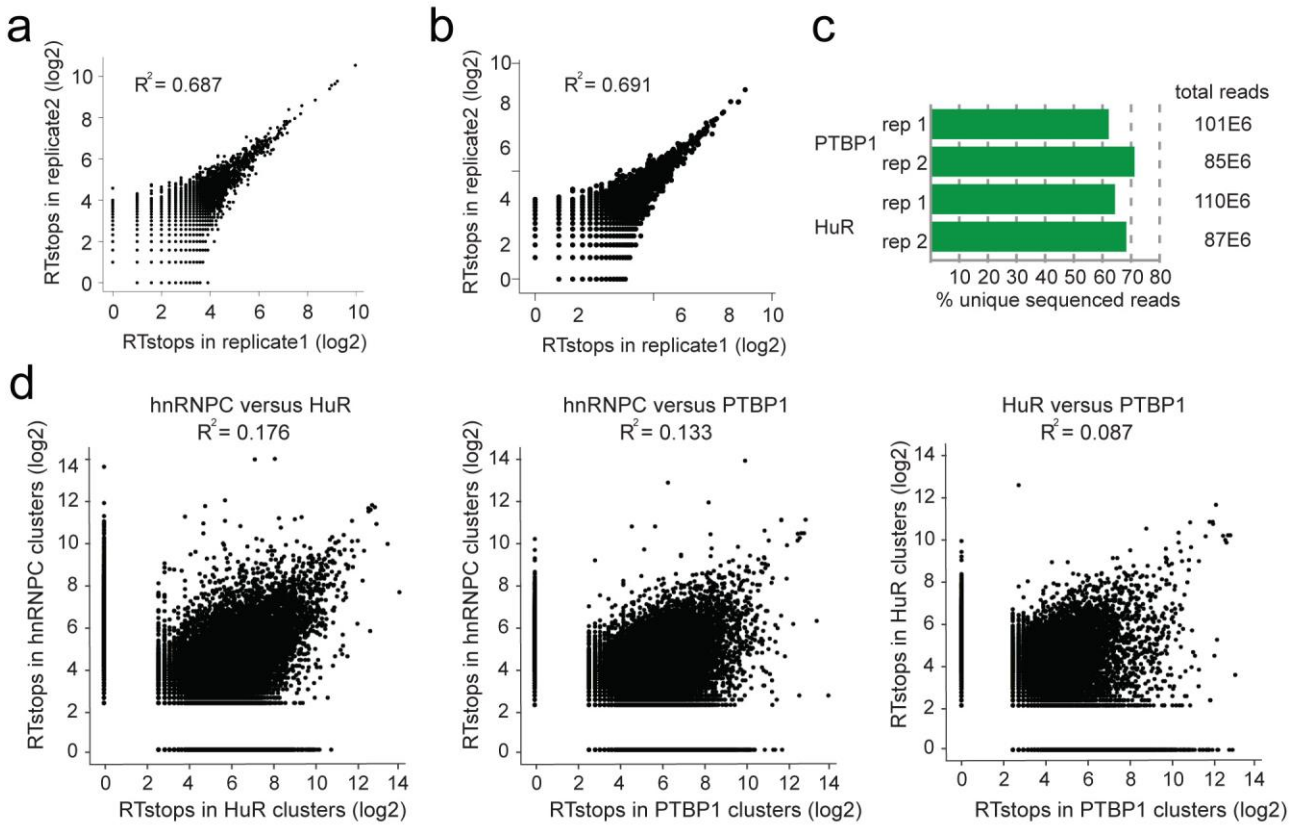
irCLIP-ligated RNA was purified from hnRNP-C-RNA complexes. 1 femtomole was used as starting material for quadruplicate cDNA synthesis reactions using Super Script III (Thermo Fisher Scientific) according to the manufacturer's protocol or TGIRT-III (InGex) reverse transcriptase (**Methods**). cDNA was purified and amplified according to irCLIP library construction (**Methods**). (**a**) Histogram of relative amplification of SuperScript III vs TGIRT-III PCR1 (**Methods**) reactions. The average Ct values of SuperScript III were used as control. 1 femtomole of IR800CW-cDNA primer was purified in triplicate with the Qiagen Nucleotide Removal Kit, the Zymo Oligo Clean and Concentrator Kit per manufacturer's instructions or with 2 volumes of AMPure beads and 5 volumes of isopropanol for 15 min. (**b**) Histogram of quantitated nitrocellulose dot blots of purified IR800CW-cDNA primer. Percent capture is the average of triplicate purifications.



Supplementary Figure 6

Controls for irCLIP experiments

(**a**) HeLa cells were untreated or subjected to the indicated doses of UVC. RNA was isolated and resolved by denaturing agarose gel electrophoresis showing high quality intact RNA for the highest dose of UV-C, 0.3J/cm^2. Odyssey CLx infrared immunoblot analyses of UV-crosslinked HeLa cell extracts showing antibody specificity and IP efficiency for (**b**) hnRNPC, (**c**) HuR and (**d**) PTBP1. Input lanes (**b-d**) were loaded at 100% of total IP. Specific bands were quantitated using LiCor Image Studio software. For IPs, 1 μg of antibody was incubated with 10μg of lysate for hnRNPC or 30μg of lysate for HuR and PTBP1. For buffer and wash conditions see **Methods**.



Supplementary Figure 7

irCLIP produces high quality data sets from greatly reduced cellular inputs

Pearson correlations of RT stops between biological replicates of 100,000 cell irCLIP experiments for (**a**) PTBP1 and (**b**) HuR. (**c**) Histogram of the unique read fractions and total clusters of the deeply sequenced biological replicates of irCLIP of PTBP1 and HuR. (**d**) Pearson correlations of the RT stops within the significant clusters (>100,000 per RBP) identified in the deeply sequenced irCLIP data sets for hnRNP-C, PTBP1, and HuR. Low R^2 values indicated highly unique clusters for each RBP.

Ultraefficient irCLIP platform for characterization of protein-RNA interactions
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Supplementary Table 1
FAST-iCLIP pipeline analysis of i/irCLIP biological replicates

RBP	hnRNP-C	hnRNP-C	hnRNP-C	HuR	PTBP1
Method	iCLIP	irCLIP	irCLIP	irCLIP	irCLIP
cellular Input	5.00E+06	1.0E+05	2.00+E04	1.00E+05	1.00E+05
	sequencing reads retained at each processing step				
raw reads (R1)	1.70E+07	1.03E+08	2.35E+07	1.01E+08	8.70E+07
raw reads (R2)	2.05E+07	1.13E+08	2.31E+07	8.43E+07	1.11E+08
no duplicates (R1)	8.14E+06	7.47E+07	1.39E+07	6.72E+07	5.42E+07
no duplicates (R2)	1.14E+07	6.22E+07	1.38E+07	5.81E+07	7.94E+07
Hg19 mapped (R1)	3.86E+06	2.45E+07	6.28E+06	2.31E+07	1.92E+07
Hg19 mapped (R2)	7.84E+06	2.10E+07	6.15E+06	2.02E+07	2.79E+07
no repeats (R1)	3.57E+06	2.19E+07	5.51E+06	2.03E+07	1.66E+07
no repeats (R2)	7.35E+06	1.91E+07	5.36E+06	1.73E+07	2.38E+07
intersect total	6.82E+05	8.02E+06	8.05E+05	7.45E+06	5.45E+06
	percentages for indicated analysis				
% unique (R1)*	48.0	72.8	59.1	66.4	62.3
% unique (R2)*	55.6	54.9	59.7	68.9	71.6
% intersect**	6.2	19.6	7.4	19.8	13.5
	significant clusters FDR<0.5 (minimun 4 RT stops)				
	~39,000	~139,000	~37,000	~107,000	~109,000

*value determined by dividing 'no duplicates' by 'raw reads'
**value determined by dividing 'intersect total' by sum of 'no repeats' for R1 and R2

Supplementary Table 2
Transcriptome wide intronic antisense Alu binding for irCLIP and iCLIP RBP clusters

			direct Alu binding (intersected +/- 0 bp)		flanking Alu binding (intersected +/- 100bp)	
		total clusters	clusters	%total	clusters	%total
iCLIP	hnRNPC 5,000,000 cells (Zarnack et. al.)	~39,000	10,423	24.9	16,283	41.0
irCLIP	hnRNPC 100,000 cells	~139,000	38,958	27.8	58,700	41.9
	hnRNPC 20,000 cells	~37,000	13,557	37.3	18,432	51.0
	HuR 100,000 cells	~107,000	16,246	14.0	27,156	23.5
	PTBP1 100,000 cells	~109,000	28,766	26.0	37,211	33.6