Supporting Information:

Structural Optimization of Polymeric Carriers to Enhance the Immunostimulatory Activity of Molecularly-Defined RIG-I Agonists

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Supplementary Data:
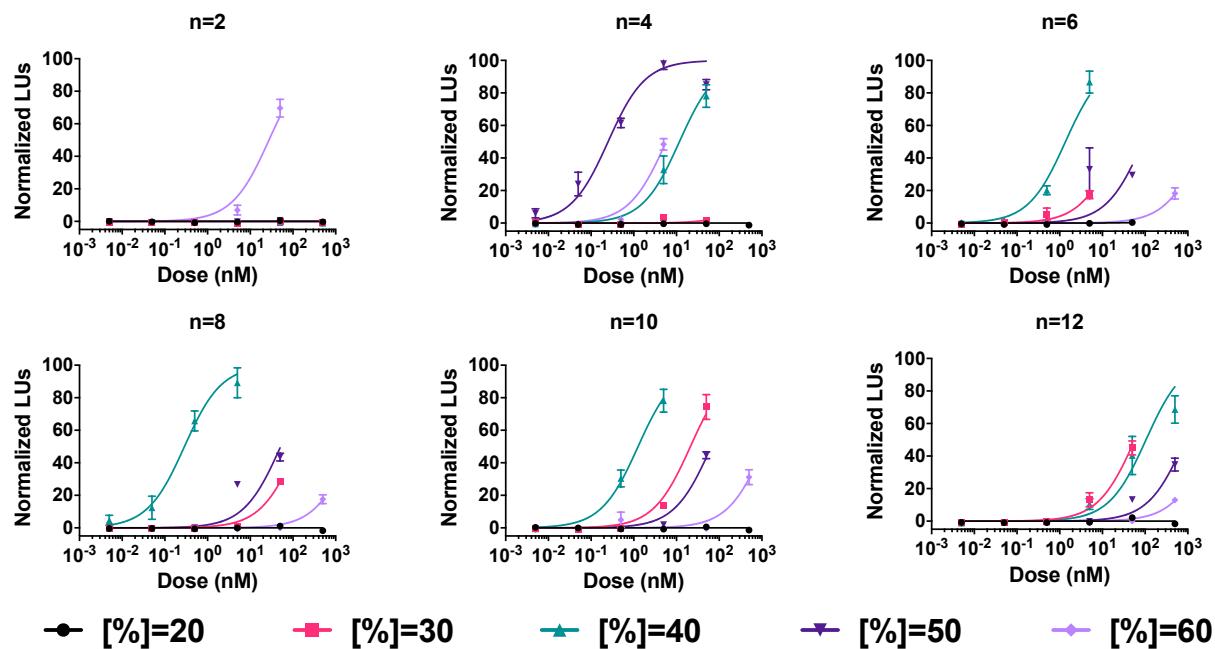
Figure S1. Dose response curves in A549 ISG reporter cells using PEG-*block*-(DMAEMA-*co*-$A_nMA[\%]$) polymers for delivery of 3pRNA.
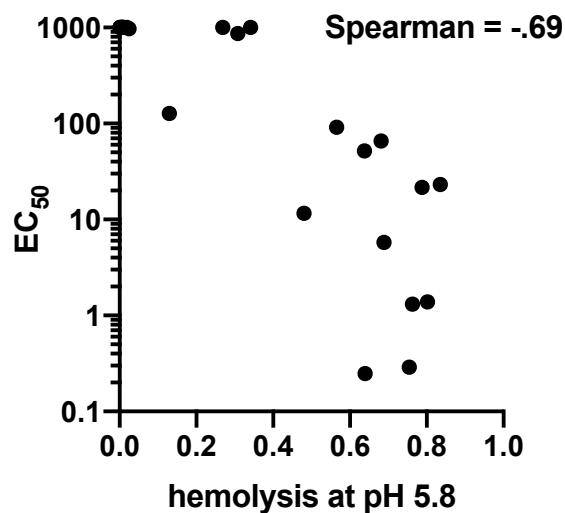
Figure S2. Spearman correlation between estimated EC_{50} of 3pRNA polymer carriers in A549 ISG cells versus fraction hemolysis at pH 5.8. EC_{50} values that could not be estimated due to lack of activity were assigned a value of 1000 nM. *P*<0.0001.
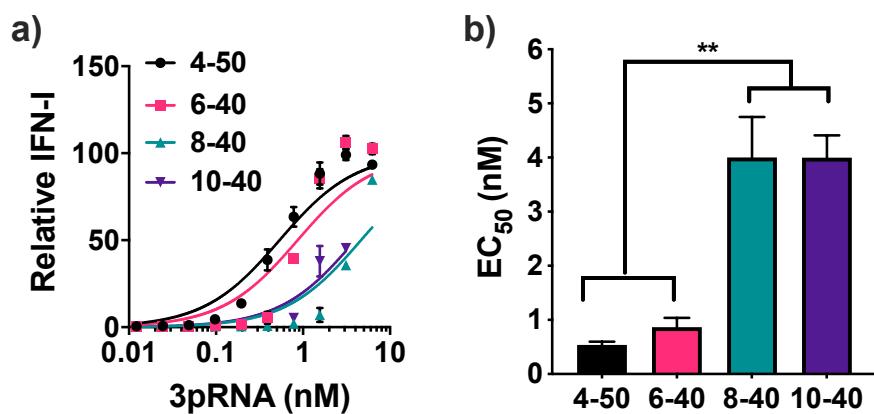

Figure S3. (a) Dose response curves of lead carriers for 3pRNA in A549 ISG reporter cells. (b) Summary of EC_{50} values for indicated NP/3pRNA complexes determined from dose response curves.
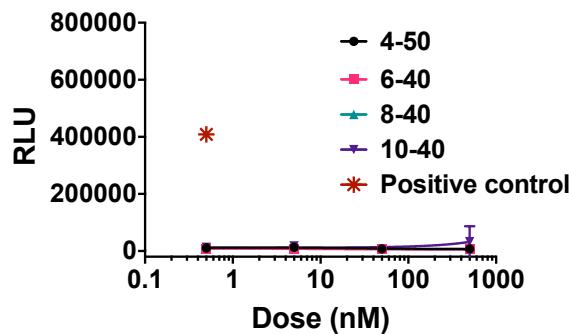
Figure S4. Dose response curves of lead carriers complexed with control OH-RNA in A549 ISG reporter cells. Positive control is 4-50 complexed with an analogous 3pRNA.
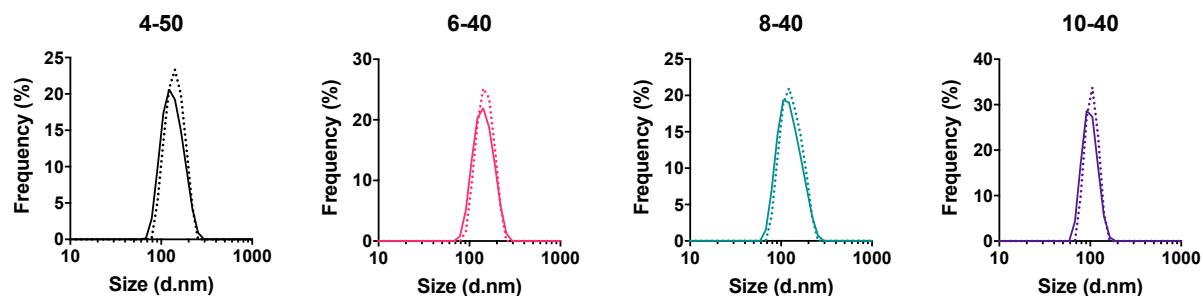

Figure S5. Size distribution of indicated NP/RNA complex measured by dynamic light scattering.
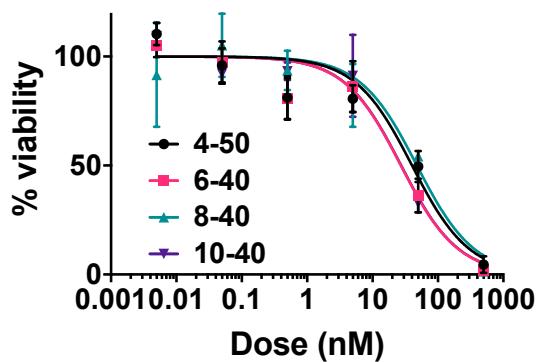
Figure S6. Viability of A549 cells after treatment with indicated NP/OH-RNA complexes for 24h.
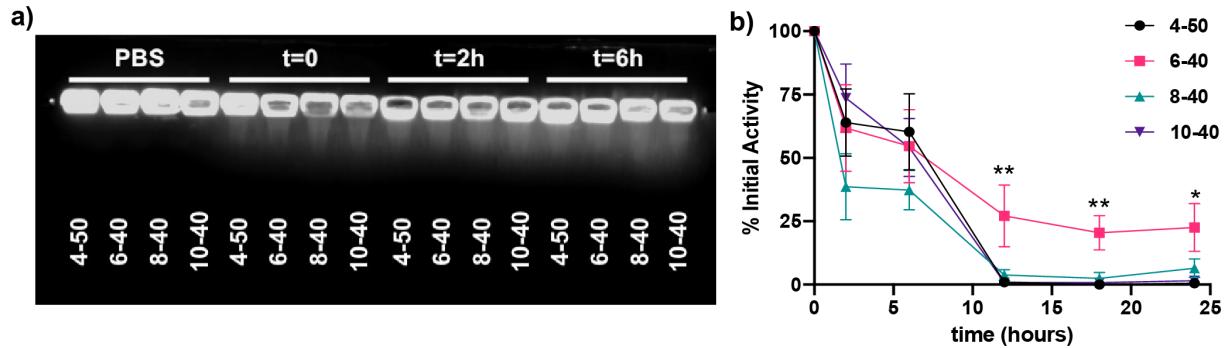
Figure S7. *In vitro* evaluation of NP/RNA stability. (a) Agarose gel electrophoresis of indicated NP/RNA complexes following incubation in 10% serum for 0, 2, or 6 h. (b) Evaluation of NP/3pRNA activity in A549-Dual cells as a function of incubation time in 80% serum, plotted as percentage of initial activity. Data are mean ± SD for three independent experiments each with N=3. *P<0.05, **P<0.01 by one-way ANOVA for 6-40 relative to other carriers.
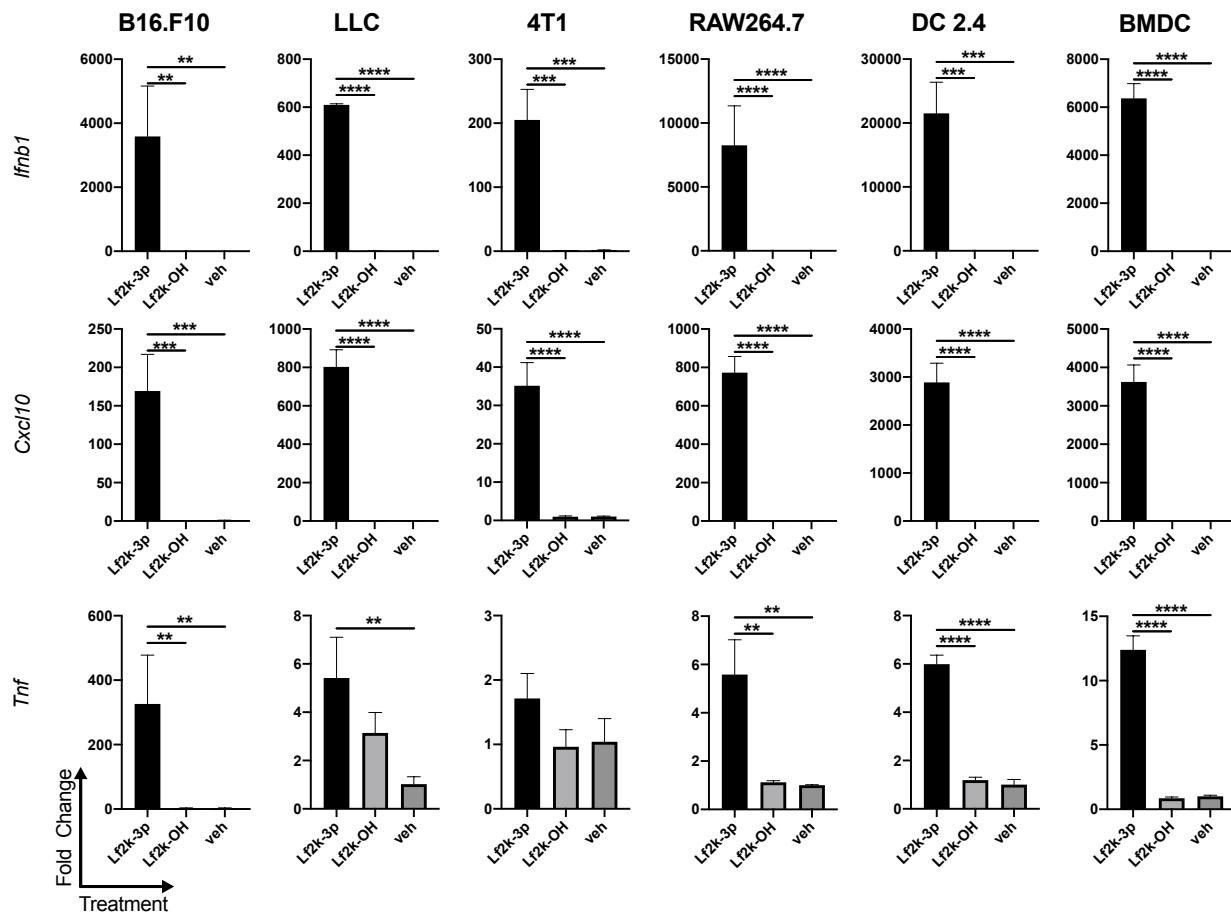
Figure S8. Evaluation of 3pRNA and negative control OH-RNA activity. The indicted cell type was treated with 3pRNA or OH-RNA was complexed with Lipofectamine 2000 (Lf2k) at 20 nM or vehicle (veh, PBS) for 6 h and expression levels of *Ifnb1*, *Cxcl10*, and *Tnf* quantified via qRT-PCR. All values plotted as mean ± SD. *P<0.05, **P<0.01, ***P<0.001,****P<0.0001 by one-way ANOVA with Tukey post-hoc test.
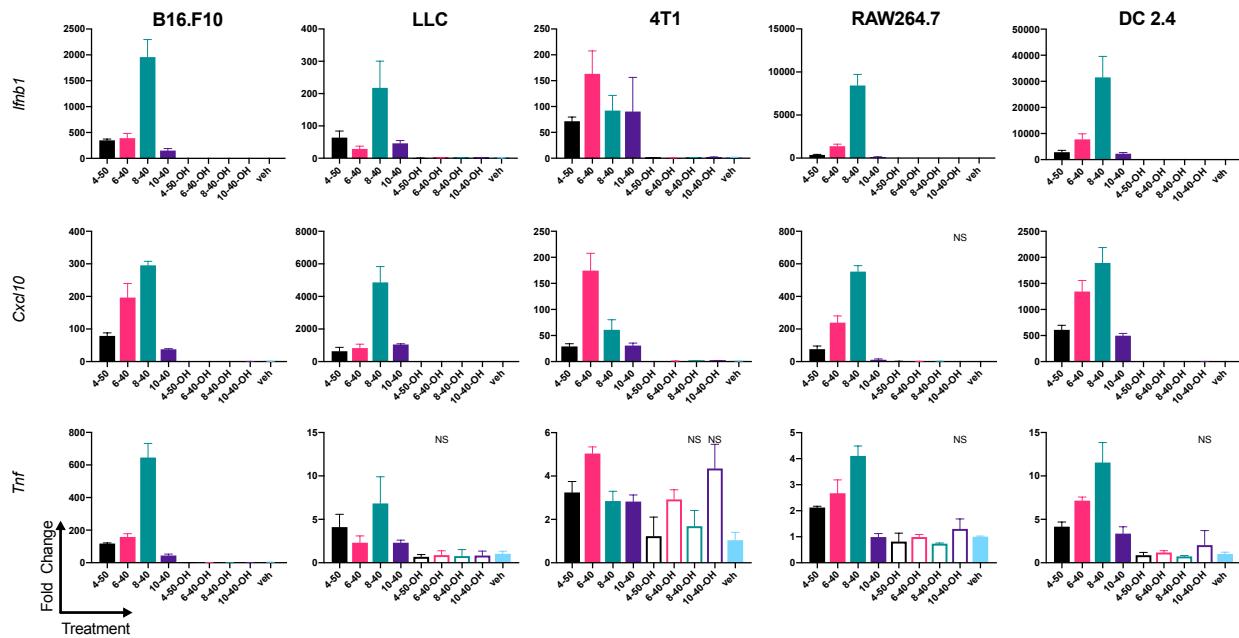
Figure S9. Evaluation of NP/3pRNA and NP/OH-RNA in cell lines. The indicted cell type was treated with 3pRNA or OH-RNA complexed with the indicated lead carrier (4-50, 6-40, 8-40, or 10-40) at 20 nM or vehicle (veh, PBS) for 6 h and expression levels of *Ifnb1*, *Cxcl10*, and *Tnf* were quantified via qRT-PCR. All values plotted as mean ± SD. All instances in which NP/3pRNA is not statistically significantly increased over NP/OH-RNA (*P*>0.05 by Student's t-test) are marked with "NS"; otherwise, NP/3pRNA is statistically significant than NP/OH-RNA with a *P*-value of at least 0.05.
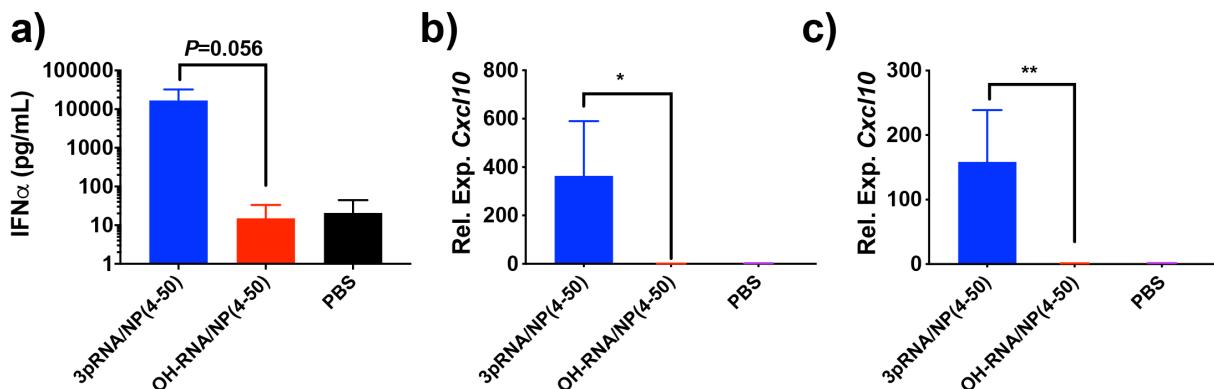
Figure S10. Mice were intravenously injected with 4-50 complexed with 3pRNA (1.25 mg/kg RNA), 4-50 complexed with negative control OH-RNA (1.25 mg/kg RNA), or PBS (vehicle) and blood, liver, and spleen were harvested five hours after injection. (a) Serum levels of IFNα measured by ELISA. Relative expression level of *Cxcl10* in the (b) liver and (c) spleen measured via qRT-PCR. All values plotted as mean ± SD. **P<0.05, **P<0.001 by one-way ANOVA with Tukey's post-hoc test; only the comparison between 3pPNA/NP(4-50) and OH-RNA/NP(4-50) is shown.
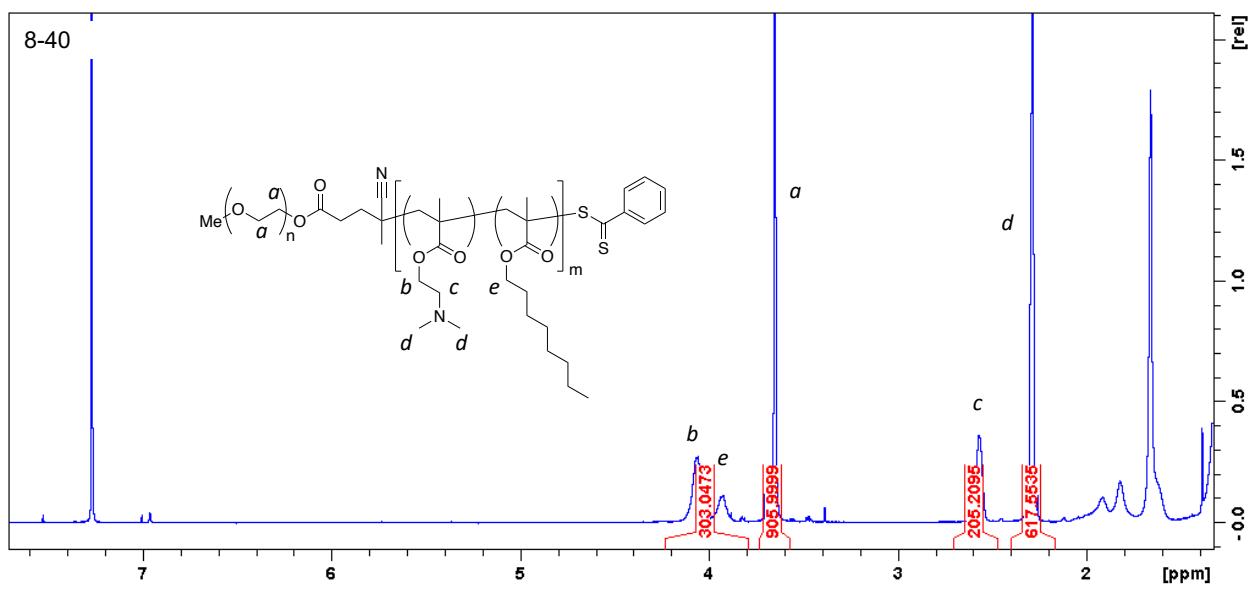
Figure S11. Representative ^{1}H NMR spectrum of 8-40. All other polymers have the same characteristic spectra.

Table S1: Statistical analysis of hemolysis data in Figure 2c – comparison between pH values

polymer	Adjusted P-value pH comparison		
	5.8 vs. 6.6	5.8 vs. 7.4	6.6 vs. 7.4
2 - 20			
2 - 30			
2 - 40			
2 - 50			
2 - 60			
4 - 20			
4 - 30	0.6749		0.6373
4 - 40	****	****	****
4 - 50	****	****	0.0015
4 - 60	****	****	0.4802
6 - 20	0.0897	0.7307	0.3662
6 - 30	****	****	****
6 - 40	****	****	0.0372
6 - 50	****	****	
6 - 60	****	****	0.8648
8 - 20	****		****
8 - 30		0.0027	0.0020
8 - 40	****	****	0.6439
8 - 50	****	****	
8 - 60	****	****	
10 - 20	0.0287	0.774	0.1440
10 - 30	****	****	****
10 - 40	****	****	0.7546
10 - 50	****	****	0.4068
10 - 60	0.7407	0.6773	
12 - 20			
12 - 30	0.8941		
12 - 40			
12 - 50	****	****	
12 - 60			

Table S2: Statistical analysis of hemolysis data in Figure 2c – comparison between polymers

polymer comparison	Adjusted P-value		
	pH 5.8	pH 6.6	pH 7.4
2-20 vs. 2-30			
2-20 vs. 2-40			
2-20 vs. 2-50			
2-20 vs. 2-60			
2-20 vs. 4-20			
2-20 vs. 4-30			
2-20 vs. 4-40	****	****	
2-20 vs. 4-50	****	****	
2-20 vs. 4-60	****		
2-20 vs. 6-20		0.0588	****
2-20 vs. 6-30	****	****	0.8461
2-20 vs. 6-40	****	0.0223	
2-20 vs. 6-50	****		
2-20 vs. 6-60	0.0011		
2-20 vs. 8-20		****	0.0008
2-20 vs. 8-30		****	
2-20 vs. 8-40	****		
2-20 vs. 8-50	****		
2-20 vs. 8-60	0.0469		
2-20 vs. 10-20		0.0017	****
2-20 vs. 10-30	****	****	
2-20 vs. 10-40	****		
2-20 vs. 10-50	****		
2-20 vs. 10-60			
2-20 vs. 12-20			
2-20 vs. 12-30			
2-20 vs. 12-40			
2-20 vs. 12-50	0.0019		
2-20 vs. 12-60			
2-30 vs. 2-40			
2-30 vs. 2-50			
2-30 vs. 2-60			
2-30 vs. 4-20			0.8458
2-30 vs. 4-30			0.1052
2-30 vs. 4-40	****	****	
2-30 vs. 4-50	****	****	
2-30 vs. 4-60	****		
2-30 vs. 6-20		0.1386	****
2-30 vs. 6-30	****	****	0.0438
2-30 vs. 6-40	****	0.0588	
2-30 vs. 6-50	****		
2-30 vs. 6-60	0.0011		
2-30 vs. 8-20		****	****
2-30 vs. 8-30		****	
2-30 vs. 8-40	****		
2-30 vs. 8-50	****		
2-30 vs. 8-60	0.0460		

2-30 vs. 10-20		0.0055	****
2-30 vs. 10-30	****	****	
2-30 vs. 10-40	****		
2-30 vs. 10-50	****		
2-30 vs. 10-60			
2-30 vs. 12-20			
2-30 vs. 12-30			
2-30 vs. 12-40			
2-30 vs. 12-50	0.0019		
2-30 vs. 12-60			
2-40 vs. 2-50			
2-40 vs. 2-60			
2-40 vs. 4-20			0.6430
2-40 vs. 4-30		0.8767	0.0439
2-40 vs. 4-40	****	****	
2-40 vs. 4-50	****	****	
2-40 vs. 4-60	****		
2-40 vs. 6-20		0.0197	****
2-40 vs. 6-30	****	****	0.0165
2-40 vs. 6-40	****	0.0067	
2-40 vs. 6-50	****		
2-40 vs. 6-60	0.0006		
2-40 vs. 8-20		****	****
2-40 vs. 8-30		****	
2-40 vs. 8-40	****		
2-40 vs. 8-50	****		
2-40 vs. 8-60	0.0271		
2-40 vs. 10-20		0.0004	****
2-40 vs. 10-30	****	****	0.7865
2-40 vs. 10-40	****		
2-40 vs. 10-50	****		
2-40 vs. 10-60			
2-40 vs. 12-20			
2-40 vs. 12-30			
2-40 vs. 12-40			
2-40 vs. 12-50	0.0009		
2-40 vs. 12-60			
2-50 vs. 2-60			
2-50 vs. 4-20			
2-50 vs. 4-30			0.7179
2-50 vs. 4-40	****	****	
2-50 vs. 4-50	****	****	
2-50 vs. 4-60	****		
2-50 vs. 6-20		0.0807	****
2-50 vs. 6-30	****	****	0.4869
2-50 vs. 6-40	****	0.0317	
2-50 vs. 6-50	****		
2-50 vs. 6-60	0.0005		
2-50 vs. 8-20		****	0.0001
2-50 vs. 8-30		****	
2-50 vs. 8-40	****		
2-50 vs. 8-50	****		

2-50 vs. 8-60	0.0235		
2-50 vs. 10-20		0.0026	****
2-50 vs. 10-30	****	****	
2-50 vs. 10-40	****		
2-50 vs. 10-50	****		
2-50 vs. 10-60			
2-50 vs. 12-20			
2-50 vs. 12-30			
2-50 vs. 12-40			
2-50 vs. 12-50	0.0008		
2-50 vs. 12-60			
2-60 vs. 4-20			
2-60 vs. 4-30			0.2791
2-60 vs. 4-40	****	****	
2-60 vs. 4-50	****	0.0001	
2-60 vs. 4-60	****		
2-60 vs. 6-20		0.1520	****
2-60 vs. 6-30	****	****	0.1374
2-60 vs. 6-40	****	0.0654	
2-60 vs. 6-50	****		
2-60 vs. 6-60	0.0007		
2-60 vs. 8-20		****	****
2-60 vs. 8-30		****	
2-60 vs. 8-40	****		
2-60 vs. 8-50	****		
2-60 vs. 8-60	0.0318		
2-60 vs. 10-20		0.0063	****
2-60 vs. 10-30	****	****	
2-60 vs. 10-40	****		
2-60 vs. 10-50	****		
2-60 vs. 10-60			
2-60 vs. 12-20			
2-60 vs. 12-30			
2-60 vs. 12-40			
2-60 vs. 12-50	0.0011		
2-60 vs. 12-60			
4-20 vs. 4-30			
4-20 vs. 4-40	****	****	0.8462
4-20 vs. 4-50	****	****	0.7179
4-20 vs. 4-60	****		0.7179
4-20 vs. 6-20		0.0499	****
4-20 vs. 6-30	****	****	
4-20 vs. 6-40	****	0.0186	
4-20 vs. 6-50	****		
4-20 vs. 6-60	0.0008		
4-20 vs. 8-20		****	0.0165
4-20 vs. 8-30		****	
4-20 vs. 8-40	****		
4-20 vs. 8-50	****		0.8952
4-20 vs. 8-60	0.0344		0.8462
4-20 vs. 10-20		0.0014	****
4-20 vs. 10-30	****	****	

4-20 vs. 10-40	****		0.4870
4-20 vs. 10-50	****		0.5650
4-20 vs. 10-60			0.8952
4-20 vs. 12-20			
4-20 vs. 12-30			
4-20 vs. 12-40			0.8462
4-20 vs. 12-50	0.0013		0.7179
4-20 vs. 12-60			
4-30 vs. 4-40	****	****	0.1054
4-30 vs. 4-50	****	0.0377	0.0595
4-30 vs. 4-60	****		0.0595
4-30 vs. 6-20			0.0002
4-30 vs. 6-30	****	****	
4-30 vs. 6-40	****		0.1765
4-30 vs. 6-50	****		0.2239
4-30 vs. 6-60	0.0012		0.2791
4-30 vs. 8-20		****	0.4114
4-30 vs. 8-30		0.0282	0.7179
4-30 vs. 8-40	****		0.2791
4-30 vs. 8-50	****		0.1375
4-30 vs. 8-60	0.0496		0.1054
4-30 vs. 10-20		0.4726	****
4-30 vs. 10-30	****	****	
4-30 vs. 10-40	****		0.0231
4-30 vs. 10-50	****		0.0320
4-30 vs. 10-60			0.1375
4-30 vs. 12-20			
4-30 vs. 12-30			0.8461
4-30 vs. 12-40			0.1054
4-30 vs. 12-50	0.0021		0.0595
4-30 vs. 12-60			0.643
4-40 vs. 4-50	0.7143	0.4185	
4-40 vs. 4-60	0.3278	****	
4-40 vs. 6-20	****	0.0007	****
4-40 vs. 6-30	****	****	0.0439
4-40 vs. 6-40	0.0005	0.0024	
4-40 vs. 6-50		****	
4-40 vs. 6-60		****	
4-40 vs. 8-20	****	0.7726	****
4-40 vs. 8-30	0.0004	0.4865	
4-40 vs. 8-40	0.0248	****	
4-40 vs. 8-50	0.7336	****	
4-40 vs. 8-60	0.2864	****	
4-40 vs. 10-20	****	0.0299	****
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4-40 vs. 10-60	****	****	
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4-40 vs. 12-30	****	****	
4-40 vs. 12-40	****	****	
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4-40 vs. 12-60	****	****	
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4-50 vs. 6-20	****		****
4-50 vs. 6-30	0.2985	****	0.0231
4-50 vs. 6-40	0.6796		
4-50 vs. 6-50		****	
4-50 vs. 6-60	0.0072	0.0003	
4-50 vs. 8-20	****	0.0002	****
4-50 vs. 8-30	****		
4-50 vs. 8-40		0.0045	
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4-50 vs. 8-60	0.0001	****	
4-50 vs. 10-20	****		****
4-50 vs. 10-30		****	0.8461
4-50 vs. 10-40		0.0007	
4-50 vs. 10-50		0.0282	
4-50 vs. 10-60	****	****	
4-50 vs. 12-20	****	****	
4-50 vs. 12-30	****	0.0002	
4-50 vs. 12-40	****	****	
4-50 vs. 12-50	0.0002	****	
4-50 vs. 12-60	****	****	
4-60 vs. 6-20	****		****
4-60 vs. 6-30		****	0.0231
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4-60 vs. 8-30	****	0.0113	
4-60 vs. 8-40			
4-60 vs. 8-50			
4-60 vs. 8-60	****		
4-60 vs. 10-20	****	0.2852	****
4-60 vs. 10-30		****	0.8461
4-60 vs. 10-40			
4-60 vs. 10-50			
4-60 vs. 10-60	****		
4-60 vs. 12-20	****		
4-60 vs. 12-30	****		
4-60 vs. 12-40	****		
4-60 vs. 12-50	****		
4-60 vs. 12-60	****		
6-20 vs. 6-30	****	****	0.0005
6-20 vs. 6-40	****		****
6-20 vs. 6-50	****	0.0527	****
6-20 vs. 6-60	0.0007	0.2852	****
6-20 vs. 8-20		****	0.7861
6-20 vs. 8-30			****
6-20 vs. 8-40	****	0.7726	****
6-20 vs. 8-50	****	0.1664	****
6-20 vs. 8-60	0.0337	0.0620	****

6-20 vs. 10-20			
6-20 vs. 10-30	****	****	****
6-20 vs. 10-40	****	0.4053	****
6-20 vs. 10-50	****		****
6-20 vs. 10-60		0.0356	****
6-20 vs. 12-20		0.1202	****
6-20 vs. 12-30		0.2157	****
6-20 vs. 12-40		0.0557	****
6-20 vs. 12-50	0.0012	0.0472	****
6-20 vs. 12-60		0.0499	****
6-30 vs. 6-40		****	0.0795
6-30 vs. 6-50	0.0108	****	0.1054
6-30 vs. 6-60	****	****	0.1374
6-30 vs. 8-20	****	****	0.6426
6-30 vs. 8-30	****	****	0.4869
6-30 vs. 8-40		****	0.1374
6-30 vs. 8-50	0.2823	****	0.0595
6-30 vs. 8-60	****	****	0.0439
6-30 vs. 10-20	****	****	0.0001
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6-30 vs. 10-40		****	0.0082
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6-30 vs. 10-60	****	****	0.0595
6-30 vs. 12-20	****	****	0.8952
6-30 vs. 12-30	****	****	0.6429
6-30 vs. 12-40	****	****	0.0439
6-30 vs. 12-50	****	****	0.0231
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6-40 vs. 8-20	****	****	****
6-40 vs. 8-30	****		
6-40 vs. 8-40		0.5427	
6-40 vs. 8-50	0.6593	0.0727	
6-40 vs. 8-60	****	0.0236	
6-40 vs. 10-20	****		****
6-40 vs. 10-30		****	
6-40 vs. 10-40		0.2157	
6-40 vs. 10-50		0.8933	
6-40 vs. 10-60	****	0.0128	
6-40 vs. 12-20	****	0.0499	
6-40 vs. 12-30	****	0.0988	
6-40 vs. 12-40	****	0.0210	
6-40 vs. 12-50	****	0.0175	
6-40 vs. 12-60	****	0.0186	
6-50 vs. 6-60	0.1922		
6-50 vs. 8-20	****	****	****
6-50 vs. 8-30	****	****	
6-50 vs. 8-40	0.5235		
6-50 vs. 8-50			
6-50 vs. 8-60	0.0079		

6-50 vs. 10-20	****	0.0015	****
6-50 vs. 10-30	0.2078	****	
6-50 vs. 10-40	0.2783		
6-50 vs. 10-50			
6-50 vs. 10-60	****		
6-50 vs. 12-20	****		
6-50 vs. 12-30	****		
6-50 vs. 12-40	****		
6-50 vs. 12-50	0.0200		
6-50 vs. 12-60	****		
6-60 vs. 8-20	0.0021	****	****
6-60 vs. 8-30	0.4308	0.0002	
6-60 vs. 8-40	****		
6-60 vs. 8-50	0.0079		
6-60 vs. 8-60			
6-60 vs. 10-20	0.0010	0.0164	****
6-60 vs. 10-30	****	****	
6-60 vs. 10-40	****		
6-60 vs. 10-50	0.0008		
6-60 vs. 10-60	0.0028		
6-60 vs. 12-20	0.0005		
6-60 vs. 12-30	0.0005		
6-60 vs. 12-40	0.0023		
6-60 vs. 12-50			
6-60 vs. 12-60	0.0010		
8-20 vs. 8-30		0.0002	0.0001
8-20 vs. 8-40	****	****	****
8-20 vs. 8-50	****	****	****
8-20 vs. 8-60	0.0744	****	****
8-20 vs. 10-20		****	0.4864
8-20 vs. 10-30	****		0.0082
8-20 vs. 10-40	****	****	****
8-20 vs. 10-50	****	****	****
8-20 vs. 10-60		****	****
8-20 vs. 12-20		****	0.0012
8-20 vs. 12-30		****	0.0002
8-20 vs. 12-40		****	****
8-20 vs. 12-50	0.0036	****	****
8-20 vs. 12-60		****	****
8-30 vs. 8-40	****	0.0032	
8-30 vs. 8-50	****	****	
8-30 vs. 8-60		****	
8-30 vs. 10-20			****
8-30 vs. 10-30	****	****	
8-30 vs. 10-40	****	0.0005	
8-30 vs. 10-50	****	0.0210	
8-30 vs. 10-60		****	
8-30 vs. 12-20		****	
8-30 vs. 12-30		0.0001	
8-30 vs. 12-40		****	
8-30 vs. 12-50	0.6511	****	

8-30 vs. 12-60		****	
8-40 vs. 8-50			
8-40 vs. 8-60	****		
8-40 vs. 10-20	****	0.1261	****
8-40 vs. 10-30		****	
8-40 vs. 10-40			
8-40 vs. 10-50			
8-40 vs. 10-60	****		
8-40 vs. 12-20	****		
8-40 vs. 12-30	****		
8-40 vs. 12-40	****		
8-40 vs. 12-50	****		
8-40 vs. 12-60	****		
8-50 vs. 8-60	0.0001		
8-50 vs. 10-20	****	0.0072	****
8-50 vs. 10-30		****	
8-50 vs. 10-40			
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8-50 vs. 12-40	****		
8-50 vs. 12-50	0.0003		
8-50 vs. 12-60	****		
8-60 vs. 10-20	0.0410	0.0018	****
8-60 vs. 10-30	****	****	
8-60 vs. 10-40	****		
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8-60 vs. 10-60			
8-60 vs. 12-20	0.0260		
8-60 vs. 12-30	0.0250		
8-60 vs. 12-40	0.0666		
8-60 vs. 12-50			
8-60 vs. 12-60	0.0426		
10-20 vs. 10-30	****	****	****
10-20 vs. 10-40	****	0.0299	****
10-20 vs. 10-50	****	0.4053	****
10-20 vs. 10-60		0.0009	****
10-20 vs. 12-20		0.0045	****
10-20 vs. 12-30		0.0106	****
10-20 vs. 12-40		0.0016	****
10-20 vs. 12-50	0.0016	0.0013	****
10-20 vs. 12-60		0.0014	****
10-30 vs. 10-40		****	0.6430
10-30 vs. 10-50		****	0.7179
10-30 vs. 10-60	****	****	
10-30 vs. 12-20	****	****	
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10-30 vs. 12-50	****	****	0.8461
10-30 vs. 12-60	****	****	
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10-60 vs. 12-50	0.0050		
10-60 vs. 12-60			
12-20 vs. 12-30			
12-20 vs. 12-40			
12-20 vs. 12-50	0.0009		
12-20 vs. 12-60			
12-30 vs. 12-40			
12-30 vs. 12-50	0.0008		
12-30 vs. 12-60			
12-40 vs. 12-50	0.0043		
12-40 vs. 12-60			
12-50 vs. 12-60	0.0017		