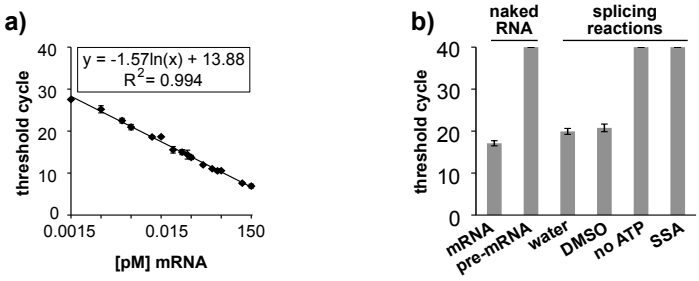
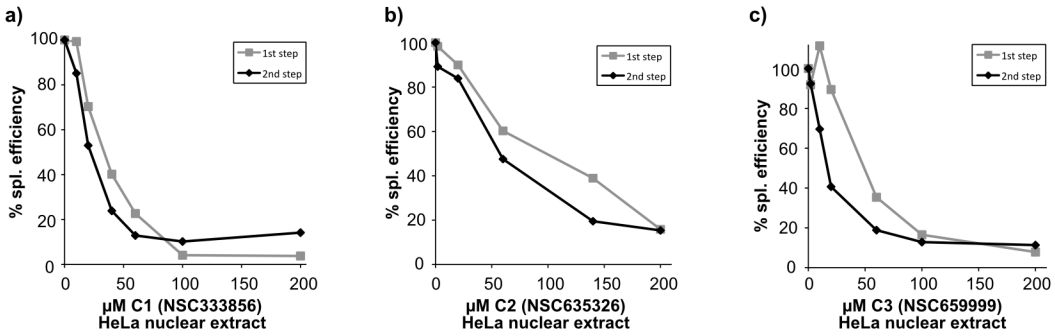
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure Legends

Supplemental Figure 1 The RT-qPCR assay has a broad dynamic range and specifically detects mRNA produced by *in vitro* splicing in HeLa nuclear extract.

a) The assay reliably detects mRNA in the low pM range. Plotted is threshold cycle (C_T) vs. concentration of synthetic mRNA in RT-qPCR. Regression analysis determined a linear range of detection over six orders of magnitude with a correlation coefficient of 0.994. The PCR efficiency is 90% (qPCR efficiency = $10^{(-1/\text{slope})}$–10).

b) Assay specificity represented another concern because in vitro splicing is not 100% efficient. A significant amount of pre-mRNA will remain in the reaction, especially if splicing is inhibited. We tested whether pre-mRNA will be also be detected with the exon-junction RT-qPCR probe. Whereas 5 nM mRNA was readily detected with a low C_T value, we see no signal for pre-mRNA at twice the amount (left part, naked RNA). Next, we tested how the assay performs with mRNA produced by *in vitro* splicing. With normal splicing reactions we consistently observed a C_T value of ~20 cycles, which is consistent with the amount of mRNA expected from uninhibited splicing. Because most small molecule libraries that we will screen for inhibitors are solubilized in DMSO, we tested whether DMSO alone affects splicing chemistry. We found that, compared to uninhibited splicing reactions, 2% DMSO did not change the C_T value significantly (right part, splicing reactions). Finally, to ensure that we can detect splicing inhibition, we examined splicing reactions supplemented with the known splicing inhibitor SSA or depleted of ATP, which is required for splicing. In both cases, no reporter fluorescence was observed after 40 cycles of qPCR, indicating that no mRNA was produced in the reaction (right part, splicing reactions).

Supplemental Figure 2 Quantification of first and second step splicing efficiency vs. inhibitor concentration for the splicing reactions shown in Figure 2a. a) C1. b) C2. c) C3. Splicing efficiency for first step is the amount of intermediates relative to total RNA and normalized to a DMSO control reaction. Splicing efficiency for second step is the amount of mRNA relative to total RNA and normalized to a DMSO control reaction.