A systematic evaluation of the design and context dependencies of massively parallel reporter assays

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Massively parallel reporter assays (MPRAs) functionally screen thousands of sequences for regulatory activity in parallel. To date, there are limited studies that systematically compare differences in MPRA design. Here, we screen a library of 2,440 candidate liver enhancers and controls for regulatory activity in HepG2 cells using nine different MPRA designs. We identify subtle but significant differences that correlate with epigenetic and sequence-level features, as well as differences in dynamic range and reproducibility. We also validate that enhancer activity is largely independent of orientation, at least for our library and designs. Finally, we assemble and test the same enhancers as 192-mers, 354-mers and 678-mers and observe sizable differences. This work provides a framework for the experimental design of high-throughput reporter assays, suggesting that the extended sequence context of tested elements and to a lesser degree the precise assay, influence MPRA results.

Spatiotemporal control of gene expression is orchestrated in part by distally located DNA sequences known as enhancers. The first enhancers were identified by cloning fragments of DNA into a plasmid with a reporter gene and promoter1–3. Transcriptional enhancement in such reporter assays continues to be widely used for evaluating whether a putative regulatory element is a bona fide enhancer. However, conventional, one-at-a-time reporter assays are insufficiently scalable to test the >1 million putative enhancers in the human genome5–8.

MPRAs modify in vitro reporter assays to facilitate simultaneous testing of thousands of putative regulatory elements5–8 per experiment. MPRAs characterize each element through sequencing-based quantification of transcribed, element-linked barcodes6–10. MPRAs have facilitated the scalable study of putative regulatory elements for goals, including functional annotation11–13, variant effect prediction14–16 and evolutionary reconstruction17,18.

Over the past decade, diverse designs for enhancer-focused MPRAs have emerged. Major differences include whether the enhancer is upstream10,11 versus within the 3′ untranslated region (UTR) of the reporter10 and whether the construct remains episomal versus integrated19. Additionally, most MPRAs test sequences in only one orientation, effectively assuming enhancer activity is independent of orientation. Finally, while sheared genomic DNA16,20, PCR amplicons12 or hybrid captured sequences13,24 have been used in MPRAs, most studies synthesize libraries of candidate enhancers on microarrays, generally limiting them to <200 base pair (bp).

Unfortunately, we have, as a field to date, largely failed to systematically evaluate how these design choices impact or bias the results of MPRAs; previous work in this vein is briefly discussed in Supplementary Note 1. Particularly as efforts to validate a vast number of putative enhancers3–8 take shape, a clear-eyed understanding of the biases and tradeoffs introduced by MPRA experimental design choices is needed. We performed a systematic comparison by testing the same 2,440 sequences for regulatory activity using nine MPRA strategies, including conventional episomal, self-transcribing active regulatory region sequencing (STARR-seq) and lentiviral designs. We further tested the same sequences in both orientations. Finally, we improved multiplex pairwise assembly25 and applied it to test differently sized versions of the same enhancers. Our results quantify the impact of MPRA experimental design choices and provide further insight into the nature of enhancers.

Results

Implementation and testing of nine MPRA strategies. We sought to systematically compare nine MPRA strategies (Fig. 1). A first strategy is related to the ‘classic’ MPRA, using the pGL4.23c vector, wherein the enhancer library resides upstream of a minimal promoter and the associated barcodes reside in the 3′ UTR of the reporter gene (pGL4)16,26. A second pair of strategies is related to STARR-seq, wherein the enhancer library resides in the 3′ UTR of the reporter gene, either as originally described (human STARR-seq; HSS)19 or using the bacterial origin of replication for transcriptional initiation (ORI)21. In both cases, we introduce barcodes immediately adjacent to the enhancers in the 3′ UTR to facilitate consistent procedures with other assays. A third set of strategies is related to LentiMPRA, wherein lentiviral integration is used to mitigate concerns about potential differences in chromatin between episomes versus chromosomes, either with the enhancer library upstream of...
the minimal promoter and the associated barcodes in the 3’ UTR of the reporter (5’/3’ wild-type (WT))\(^{18}\), the enhancer library upstream of the minimal promoter and the barcodes in the 5’ UTR of the reporter (5’/5’ WT), or both the enhancer library and the barcodes in the 3’ UTR of the reporter (3’/3’ WT). The 5’/5’ WT design was developed to address distance-dependent template switching before lentiviral integration\(^{27,28}\), as it reduces the distance between the enhancer and barcode from 801 to 102 bp. The 3’/3’ WT design is analogous to STARR-seq, but is integrated into the genome and also addresses template switching by positioning the enhancer and barcode immediately adjacent to one another. A fourth set of designs are identical to the three lentiMPRA designs, except that the vector harbors a mutant (MT) integrase, such that the constructs remain episomal (5’/3’ MT, 5’/5’ MT, 3’/3’ MT)\(^{18}\).

For a common set of sequences to test, we turned to a previously developed library\(^{18}\) consisting of 2,236 candidate enhancer sequences based on HepG2 chromatin immunoprecipitation sequencing (ChIP-seq) peaks, along with 204 controls (Supplementary Table 1). Of these, 281 overlapped promoters (±1 kb of the transcription start sites (TSSs)) of protein-coding genes. The controls consist of synthetically designed sequences that previously demonstrated enhancer MPRA activity (100 positives) or lack thereof (100 negatives)\(^{18}\) in HepG2 cells, along with 2 positive and 2 negative controls derived from endogenous sequences that were previously validated with luciferase assays\(^{18}\). All sequences were 171 bp and synthesized from endogenous sequences that were previously validated with microarray assays. A 15-bp degenerate barcode was appended during PCR amplification and amplicons were cloned to the HSS vector. The enhancer/barcode region of the HSS library was amplified and used for two purposes; first, it was sequenced to link barcodes to enhancers; second, the amplicons were cloned at high complexity into other vectors to create libraries for the remaining eight MPRA designs (Supplementary Fig. 1). As such, the relative abundances of enhancers and barcodes, as well as the enhancer–barcode associations, were consistent across all MPRA libraries. Cloning details and references for each of the nine assay designs are provided in the Methods.

Plasmid libraries were transfected into HepG2 cells in triplicate (three different days). LentiMPRA libraries were packaged with either WT or MT integrase lentivirus and infected into HepG2 in triplicate (three different days). We extracted DNA and RNA, amplified barcodes via PCR and PCR with reverse transcription (RT), respectively, and sequenced amplicons to generate barcode
counts (Fig. 1). An activity score for each element was calculated as the log of the normalized count of RNA molecules from all barcodes corresponding to the element, divided by the normalized number of DNA molecules from all barcodes corresponding to the element (Supplementary Table 2). For each of the 27 experiments (nine assays with three replicates), only barcodes observed in both RNA and DNA were considered. For 26 of 27 experiments (all but 3‘/3′ MT replicate 1), the median number of barcode counts per element was >100 (Supplementary Fig. 2).

Comparing results from different MPRA designs. We first sought to evaluate the technical reproducibility of each assay. Most assays were highly correlated between the three replicates. Specifically, intra-assay Pearson correlations for pairwise comparisons of activity scores of replicates exceeded 0.90 for all assays except for 5‘/3′ MT (mean r=0.87) and 3‘/3′ MT (mean r=0.54) assays (Fig. 2a and Supplementary Fig. 3a). We also confirmed correlations for 5‘/3′ WT and 5‘/3′ MT between this and our previous study32 (r=0.92 for 5‘/3′ WT and r=0.81 for 5‘/3′ MT; Supplementary Fig. 3b).

We next sought to compare the results of the various assay designs to one another. We calculated the average activity score for each element across all technical replicates of a given assay (Supplementary Table 2) and then compared the assays to one another. Six of the nine assays demonstrated inter-assay Pearson and Spearman correlations of >0.7 with all other members of this group (Fig. 2b and Supplementary Figs. 4 and 5). These were the ORI and pGL4, together with both WT and MT versions of the 5‘/3′ and 3‘/3′ assays. The remaining three assays (3‘/3′ MT, 3‘/3′ WT and HSS) did not show good agreement with the other six assays, nor with one another.

As a different approach to compare assays, we subjected activity scores from all 27 experiments (nine assays with three replicates) to principal component analysis (Fig. 2c). The aforementioned six assays with inter-assay correlations of >0.7 clustered closely to one another. Notably, principal component 1 (PC1) tended to separate the assays wherein the enhancer resides upstream of the minimal promoter (5‘/3’, 5‘/3’, and pGL4) from those wherein it resides 3‘ of the reporter gene (3‘/3’, HSS and ORI). In contrast, principal component 2 (PC2) tended to separate lentiviral designs (5‘/3’, 5‘/3’ and 3‘/3’) from plasmid-based designs (pGL4, HSS and ORI). This suggests systematic differences in the enhancer activity measurements that relate to aspects of MPRA design. It also highlights that the location of the candidate enhancer on the plasmid backbone plays a larger role in differential activity than does the episomal versus integrated aspect of the assay.

Next, we examined the dynamic range of activity scores (Fig. 2d). Of note, 3‘/3’ MT was removed from further analyses due to comparatively poor technical reproducibility (mean r=0.54). The classic enhancer reporter vector (pGL4) and the promoterless STARR-seq assay (ORI) exhibited the greatest dynamic range, with pGL4 showing the largest separation between positive and negative controls (two-sided t-statistic = 37.46). Among the lentiviral assays, the 5‘/5’ WT design exhibited the greatest dynamic range and separation of controls (two-sided t-statistic = 30.92).

We generated lasso regression models based on 915 biochemical, evolutionary, and sequence-derived features (Supplementary Tables 3 and 4) using tenfold cross-validation. We were able to predict enhancer activities for the six aforementioned assays (Pearson r ranging from 0.59 for 5‘/3’ WT to 0.71 for pGL4) (Supplementary Fig. 6a,b). In general, strong enhancers tended to be underpredicted by the model, whereas weak enhancers tended to be overpredicted.

Many of the top coefficients fitted by these models correspond to ChIP-seq signal or sequence-based binding site predictions for transcriptional activators, coactivators and repressors (Supplementary Fig. 6c,d and Supplementary Table 5). We caution that the interpretation of feature selection and coefficient-based ranking is inherently limited by substantial multicollinearity among features (Supplementary Table 4), which in turn limits the determination of which features are mechanistically or causally involved. Potential reasons for inter-feature correlations are summarized in Supplementary Note 2.

We next sought to ask whether we could predict differences in enhancer activity between the assays on the basis of the same 915 features. For models predicting pairwise differences between the results of the pGL4, 5‘/5’ WT, 3‘/3’ WT and ORI assays, we were able to achieve correlations of 0.4–0.5 (Fig. 3a and Supplementary Fig. 7a). We were particularly interested in whether features corresponding to RNA-binding proteins and splicing factors would be especially predictive of promoterless STARR-seq (ORI) or 3‘/3’ WT results, as in these assays the enhancer itself is included in the 3‘ UTR. Indeed, SRSF1/2, BRUNOL4, PTBP1, PPRC1, KHDRBS2, SYNCRIP and MBNL1, which are known to modulate mRNA stability and splicing, predict differences in measured activity in ORI or 3‘/3’ WT versus 5‘/5’ WT or pGL4 (Fig. 3b,c, Supplementary Fig. 7b,c and Supplementary Table 5). Of note, SRSF1/2, PTBP1, PPRC1, SYNCRIP and MBNL1 are all expressed in liver22 and could therefore influence MPRA results in HepG2. Additionally, several promoter-binding proteins (TEAD1, TEAD3, NRSF1, JUN and YY1), all expressed in the liver, favor pGL4 and 5‘/5’ WT, whereas CCAAT-enhancer-binding proteins favor HSS and ORI. This may correspond to a tradeoff wherein conventional MPRAis are biased toward testing for promoter-like activity, whereas STARR-seq MPRAis are biased by mRNA stability and splicing factors.

Next, we examined differences between episomal versus integrated assays. We note that FOXP1 is more predictive of integrated activity, while ETS-variant transcription factors are more predictive of episomal activity, suggesting that these or correlated factors play a differential role in episomal versus integrated contexts (Fig. 3b,c and Supplementary Fig. 7b,c).

Notably, general transcriptional activity, as measured by cap analysis gene expression (CAGE)31, was among the most predictive features of the 3‘/3’ WT assay (Supplementary Fig. 6c). This is the only assay where the tested elements are both genomically integrated and distally located from the promoter, this observation suggests that CAGE-based transcriptional activity may be a good predictor of distal enhancer activity12,13.

Enhancer activity is largely, but not completely, independent of sequence orientation. We next set out to test a key aspect of the canonical definition of enhancers, that they function independently of their orientation with respect to the promoter. We directionally cloned 2,336 sequences (2,236 candidates described above extended out to a 192-bp genomic reference sequence, along with 50 positive and 50 negative controls from Vockley et al.13), in both orientations into the pGL4 vector, pooled these libraries, and transfected HepG2 cells in quadruplicate (Fig. 4a). The median number of barcode counts per element was >100 (Supplementary Fig. 8) and the measured activities were reproducible (Pearson r = 0.98; Fig. 4b, Supplementary Fig. 9 and Supplementary Table 6). Notably, enhancer activities for the same elements cloned in forward versus reverse orientation to the pGL4 vector were also highly correlated (mean r = 0.88) but less so than same-orientation comparisons (>0.98; Fig. 4b). This suggests that enhancer activity in reporters is largely, but not completely, independent of orientation.

In contrast with enhancers, promoters are established to be directional14,15. Overall, 266 of 281 promoter-overlapping elements were successfully measured in both orientations. We tested whether these behaved differently than 1,953 more distally located elements. Indeed, the promoter-overlapping sequences exhibited greater differences in activity between the two orientations than distal elements, supporting the conclusion that they contain signals to promote transcription in an asymmetric fashion (Fig. 4c,d).
Appending sequence context leads to differences in the results of MPRAs. Most MPRAs use array-synthesized libraries that are, for technical reasons, limited in length, typically to fewer than 200 bp. To evaluate the impact of this length restriction, we designed 192-bp (‘short’), 354-bp (‘medium’) and 678-bp (‘long’) versions of our candidate enhancer library, centered at the same genomic position and corresponding to the equivalent 2,236 candidate enhancers tested above (including more flanking sequence from reference genome; Supplementary Table 1). We also included 50 high- and low-scoring putative elements from Vockley et al.12 in the short and medium libraries (excluded from long libraries because they were all shorter than 678 bp).

The 192-bp versions of these candidate enhancers were synthesized directly on a microarray; sequencing showed a 100% yield (2,336 of 2,336) and a 3.8-fold interquartile range (IQR) for relative abundance (Supplementary Fig. 10a). To generate 354-bp versions, we performed our previously published multiplex pairwise assembly on overlapping pairs of array-synthesized 192-bp fragments (95% yield (2,241 of 2,336); 4.9-fold IQR; Supplementary Fig. 10a). Finally, to generate the 678-bp versions, we developed
Fig. 3 | Predictive modeling of the ratios and differences between MPRA methods. a, Pearson and Spearman correlation coefficients for tenfold cross-validated (CV) predictions derived from lasso regression models and the observed RNA/DNA ratios, for each of the seven indicated differential comparisons tested. Also indicated are the Pearson (r) and Spearman (ρ) correlation values. b, The top ten coefficients derived from lasso regression models trained on the full dataset to predict observed differences in the indicated pairs of MPRA methods. Features with the extensions .1, .2, etc. allude to redundant features or replicate samples. c, Pearson correlation matrix between the union of all top ten features from b, shown as rows and other features sharing a Pearson correlation either ≤−0.8 or ≥0.8, shown as columns. Feature names are colored according to the origin of the feature as shown in the boxed key. Hierarchical clustering was used to group features exhibiting similar correlation patterns.

a ‘two-round’ version of MPA that we call hierarchical multiplex pairwise assembly (HMPA) (Supplementary Figs. 10b and 11). HMPA of overlapping pairs of array-synthesized 192-bp fragments yielded overlapping pairs of 354-bp fragments, which were further assembled to generate 678-bp fragments (84% yield (1,887 of 2,236); 27.9-fold IQR; Supplementary Fig. 11a). We verified a subset of our long enhancers with PacBio sequencing (Supplementary Fig. 10c,d; chimera rate of 16.5%).

We cloned all three libraries into the pGL4 vector, then pooled and transfected them in quadruplicate to HepG2 cells (Fig. 5a and Supplementary Table 7). Requiring each element to be detected with at least ten unique barcodes, there were 651 candidate enhancers tested at all three lengths. Technical replicates within any given length class were highly reproducible, albeit modestly less so for long elements (mean Pearson r = 0.94; Fig. 5b and Supplementary Figs. 12 and 13). However, there was less agreement for the same candidate enhancers tested at different lengths (short versus medium, mean r = 0.78; medium versus long, mean r = 0.67; short versus long, mean r = 0.53; Fig. 5b,c).

Finally, we observed that the positive control sequences were significantly more active than the negative controls when tested as either 192-bp or 354-bp fragments (P < 0.01, Wilcoxon signed-rank test; Fig. 5d).

We chose ten MPRA-active candidate enhancers to test in individual luciferase assays: five that showed differential activity between their long and medium forms (cyan; Supplementary Fig. 14a) and five that did not (green; Supplementary Fig. 14a). Of the five that did not show differential activity, three were active in the luciferase assay (2–4), all concordant with MPRA results (Supplementary Fig. 14b–d). Of the five that did not show differential activity in the MPRA, all were active in the luciferase assay in at least one form and four had differential activity, possibly due to greater sensitivity of the luciferase assay or subtle differences between the constructs (Supplementary Fig. 14b–d). We also tested versions of all ten of these MPRA-active candidates in their long form but with the middle 354 bp deleted; all of these showed insignificant (n = 8) or reduced (n = 2) activity in the luciferase assay (Supplementary Fig. 14b). Overall, these results highlight the relevance of the lengths and boundaries of elements tested in MPRAs in influencing measured activity.
We trained lasso regression models to predict activities using features, which were re-computed for each of the three size classes (Fig. 6a, Supplementary Fig. 15 and Supplementary Table 4). The lower performance of the model for the long element library is possibly consequent to its fewer sequences, its lower technical reproducibility or an increase in the effect of nonlinear interactions between features that reduce predictive performance. Known predictors of enhancer activity were consistently present in the top coefficients, although their relative rankings differed depending on the size class being examined (Supplementary Fig. 15c and Supplementary Table 5). Next, we sought to explicitly model how differences in predicted factor binding might explain differences in enhancer activity, as measured by different pairs of size classes. For example, in attempting to explain observed activity differences in long versus short elements, we computed a set of features as the differences in predicted binding or measured ChIP-seq signal, between the long element and corresponding short element (for example, ΔARID3A = ARID3A_long − ARID3A_short). Many of the top features originated from sequence-based differences in predicted binding in the extra genomic context surrounding the core element. Features consistently observed to explain activity differences in longer elements include RPC155, the catalytic core and largest component of RNA polymerase III; Jun and FOS, components of the AP-1 complex; ATF2, EZH2 and HDAC1/2, core histone-modifying enzymes; and the transcription factors ARID3A, DRAP1 and SP1/2/3 (Fig. 6b,c, Supplementary Fig. 16 and Supplementary Table 5).

**Discussion**

Over the past decade, MPRAs have enabled researchers to functionally test large numbers of DNA sequences for regulatory activity and in the process address numerous biological questions. While different groups utilize various backbones and assay designs, there has been no systematic comparison of how these different strategies influence results.

Here, we have sought to perform a systematic comparison of major MPRA strategies and to concurrently investigate the consequences of key design choices such as the assay, element orientation and element length. We generally observe concordance between different MPRA designs, albeit to varying degrees. Six of the nine assays exhibited both technical reproducibility as well as reasonable agreement with one another (pGL4, ORI, 5′/5′ WT, 5′/5′ MT, 5′/3′ WT and 5′/3′ MT). Furthermore, as we previously showed for the 5′/3′ WT and 5′/3′ MT assays, enhancer activities as measured by MPRAs are reasonably well predicted by models based on primary sequence together with biochemical measurements at the corresponding genomic locations. Taken together, our results...
support a view wherein diverse MPRAs are all measuring enhancer activity, but design differences (such as integrated versus episomal; 5′ versus 3′ location of the enhancer) influence the results to a modest degree. For example, features influencing mRNA stability and splicing favor assays with the enhancer transcribed in the 3′ UTR (ORI and 3′/3′ WT), whereas promoter-binding transcription factors favor assays with the enhancer upstream of the promoter (pGL4 and 5′/5′ WT).

Overall, our results support a preference for three of the nine MPRA designs evaluated here (pGL4, ORI and 5′/5′ WT), which all had reasonable inter-assay correlations. The pGL4 assay has the advantage of representing the ‘classic’ enhancer reporter assay design, had the greatest dynamic range and was the most predictable with our lasso regression, but had the disadvantages of being episomal rather than integrated and of confounding enhancer activity with possible effects from promoter-binding proteins. The ORI assay (promoterless STARR-seq) has the advantage of eliminating the need to associate barcodes, potentially allowing for greater library complexities, and has a large dynamic range, but has the disadvantages of confounding enhancer activity with possible effects on messenger RNA splicing and/or stability, and also of being episomal rather than integrated. The 5′/5′ WT assay has the advantage of being integrated rather than episomal and, among lentiviral assays, mitigates the template switching issue by minimizing the distance between the enhancer and barcode. However, template switching still occurs to some degree, the assay exhibits a lower dynamic range than pGL4 or ORI assays and has similar potential for bias from promoter-binding proteins as pGL4.

A caveat of our HSS and ORI experiments is that by incorporating a barcode downstream of the enhancer, we introduced the possibility that barcode counts include short transcripts initiating within the candidate enhancer itself. Further exploration of this potential confounder, including additional experiments, is summarized in Supplementary Note 3.

**Fig. 5** Including additional sequence context around tested elements leads to differences in the results of MPRAs. **a**, Experimental schematic. The 192-bp, 354-bp and 678-bp libraries were synthesized, assembled and cloned into the pGL4 backbone. These were pooled and transfected into HepG2 cells in quadruplicate. nt, nucleotide. **b**, Beeswarm plot of the Pearson correlation values corresponding to each of the six possible pairwise comparisons among the four replicates. The correlations are computed between observed enhancer activity values for elements measured in each of the three possible size classes. **c**, Scatter-plots of the average activity score of each element, comparing short versus medium, medium versus long, and short versus long versions of each element, and restricting to elements detected with at least ten unique barcodes at both lengths (n). **d**, Violin plot displaying the distribution of average log2(RNA/DNA) ratios for short, medium and long versions of the elements tested, as well as for positive and negative controls at short and medium lengths.
Another key finding is our confirmation that the activity of enhancers, is largely, but not completely, independent of orientation, at least as measured for our subset of candidate enhancers tested using the pGL4 vector. This is of course part of the original definition of enhancers\(^1\), but efforts to systematically test the validity of this assumption across a large number of sequences have been limited\(^{16,26,32}\). Previously, a subset of preinitiation-complex-bound enhancers were shown to have strong orientation-dependent activity, highlighting that these trends may be influenced by the choice of elements tested\(^{16}\). Candidate enhancer sequences derived from the vicinity of TSSs exhibited greater directionality, consistent with a subset of these bearing features of oriented promoters.

Finally, we developed improved methods to efficiently assemble longer DNA fragments from array-synthesized oligonucleotides and applied them to evaluate the extent to which including additional sequence context around tested elements impacts MPRA results. We successfully assembled 95% of 2,336×354-bp targets using MPA, compared to just 71% of 2,271×192–252-bp targets in our original description of the method\(^{25}\). Moreover, our HMPA is a protocol that in vitro assembles thousands of sequences, each over 600 bp, as a single library. In this manuscript, we synthesized >600 elements, each 678 bp, for 1–2% of what it would have cost from commercial vendors. Unlike potential alternatives, the method does not require specialized equipment, making it more widely accessible\(^{16}\).

The sub-200-bp length of sub-sequences typically tested is a choice related to the technical limits of microarray-based synthesis. In the genome, there are no such limits and it remains unclear what the appropriate ‘enhancer size’ is to test in MPRA’s and whether this choice matters. To evaluate this, we tested candidate enhancers at three different lengths. We observed correlations between the same elements tested at all lengths, but these correlations clearly drop off as a function of length difference. At the extreme, the activities of 678-bp versus 192-bp versions of the same candidate enhancers were more poorly correlated than nearly all of our inter-assay comparisons (Pearson \(r = 0.53\), Spearman \(\rho = 0.46\)). Furthermore, these data suggest that the longer sequences are adding biologically relevant signal, as features corresponding to relevant transcription factors explain differences in activity of longer versus shorter sequences.

### Fig. 6 | Predictive modeling of factors dependent on element size

\(a\) Pearson and Spearman values between the tenfold CV predictions and observed values for each of the three size classes tested. \(b\) The top ten coefficients derived from lasso regression models trained on the full dataset to predict observed values from the differential size comparisons indicated. Features with the extension ‘1’, ‘2’, etc. allude to redundant features or replicate samples. \(c\) Pearson correlation matrix between the union of all top ten features from \(b\), shown as rows and other features sharing a Pearson correlation either \(\leq 0.8\) or \(\geq 0.8\), shown as columns. Feature names are colored according to the origin of the feature as shown in the boxed key. Hierarchical clustering was used to group features exhibiting similar correlation patterns.
For example, a feature corresponding to RPC155, the catalytic subunit of RNA polymerase III, is the strongest coefficient separating the 678-bp constructs from the 192-bp and 354-bp constructs and also one of the stronger coefficients separating the 354-bp from 192-bp constructs. Although it is challenging to offer strict guidance in the absence of in vivo ground truth, we recommend testing longer sequences when possible.

In conclusion, we set out to rank the relative contribution of assay design, orientation and length on the results of MPFRAs. Our results suggest a degree of caution in interpreting the results of all MPFRAs, as they are all subject to influence by aspects of the assay design. We found that sequence length had the greatest effect, followed by assay design and finally orientation. Although MPFRAs of high-complexity genome-wide fragment libraries are not length limited\textsuperscript{27,28}, MPFRAs of designed libraries largely still are. For designed libraries in particular, further work is necessary to develop or improve methods such as HMPA to facilitate the construction of complex, uniform MPRA libraries of longer sequences, as well as to further explore the optimal parameters of element design (such as length and centering).

Online content
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References
1. Banerji, J., Rusconi, S. & Schaffner, W. Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. Cell 27, 299–308 (1981).
2. Moreau, P. et al. The SV40 72 base repair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. Nucleic Acids Res. 9, 6047–6068 (1981).
3. Banerji, J., Olson, L. & Schaffner, W. A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. Cell 33, 729–740 (1983).
4. Neuberger, M. S. Expression and regulation of immunoglobulin heavy chain genes transfigured into lymphoid cells. EMBO J. 2, 1373–1378 (1983).
5. Bernstein, B. E. et al. The NIH roadmap epigenomics mapping consortium. Nat. Biotechnol. 28, 1045–1048 (2010).
6. Kawaji, H., Kasukawa, T., Forrest, A., Carninci, P. & Hayashizaki, Y. The FANTOM5 collection, a data series underpinning mammalian transcriptome atlases in diverse cell types. Sci. Data 4, 170113 (2017).
7. ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. Nature 489, 57–74 (2012).
8. ENCODE Project Consortium. A user’s guide to the encyclopedia of DNA elements (ENCODE). PLoS Biol. 9, e1001046 (2011).
9. Patwardhan, R. P. et al. High-resolution analysis of DNA regulatory elements by synthetic saturation mutagenesis. Nat. Biotechnol. 27, 1173–1175 (2009).
10. Patwardhan, R. P. et al. Massively parallel functional dissection of mammalian enhancers in vivo. Nat. Biotechnol. 30, 265–270 (2012).
11. Melnikov, A. et al. Systematic dissection and optimization of inducible enhancers in human cells using a massively parallel reporter assay. Nat. Biotechnol. 30, 271 (2012).
12. Vockley, C. M. et al. Massively parallel quantification of the regulatory effects of noncoding genetic variation in a human cohort. Genome Res. 25, 1206–1214 (2015).
13. Tewhey, R. et al. Direct identification of hundreds of expression-modulating variants using a multiplexed reporter assay. Cell 172, 1132–1134 (2018).
14. Ulirsch, J. C. et al. Systematic functional dissection of common genetic variation affecting red blood cell traits. Cell 165, 1530–1545 (2016).
15. Liu, S. et al. Systematic identification of regulatory variants associated with cancer risk. Genome Biol. 18, 194 (2017).
16. Arnold, C. D. et al. Genome-wide quantitative enhancer activity maps identified by STARR-seq. Science 339, 1074–1077 (2013).
17. Kwasnieski, J. C., Fiore, C., Chaudhari, H. G. & Cohen, B. A. High-throughput functional testing of ENCODE segmentation predictions. Genome Res. 24, 1595–1602 (2014).
18. Inoue, F. et al. A systematic comparison reveals substantial differences in chromosomal versus episonal encoding of enhancer activity. Genome Res. 27, 38–52 (2017).
19. Klein, J. C. et al. Functional testing of thousands of osteoarthritis-associated variants for regulatory activity. Nat. Commun. 10, 2434 (2019).
20. Arnold, C. D. et al. Quantitative genome-wide enhancer activity maps for five Drosophila species show functional enhancer conservation and turnover during cis-regulatory evolution. Nat. Genet. 46, 685–692 (2014).
21. Klein, J. C., Keith, A., Agarwal, V., Durham, T. & Shendure, J. Functional characterization of enhancer evolution in the primate lineage. Genome Biol. 19, 99 (2018).
22. Muerdter, F. et al. Resolving systematic errors in widely used enhancer activity assays in human cells. Nat. Methods 15, 141–149 (2018).
23. Vanhille, L. et al. High-throughput and quantitative assessment of enhancer activity in mammals by CapStarr-seq. Nat. Commun. 6, 6905 (2015).
24. Wang, X. et al. High-resolution genome-wide functional dissection of transcriptional regulatory regions and nucleotides in human. Nat. Commun. 9, 5380 (2018).
25. Klein, J. C. et al. Multiplex pairwise assembly of array-derived DNA oligonucleotides. Nucleic Acids Res. 44, e43 (2016).
26. Kircher, M. et al. Saturation mutagenesis of disease-associated regulatory elements. Nat. Commun. 10, 3583 (2019).
27. Hill, A. J. et al. On the design of CRISPR-based single-cell molecular screens. Nat. Methods 15, 271–274 (2018).
28. Sack, L. M., Davoli, T., Xu, Q., Li, M. Z. & Elledge, S. J. Sources of error in mammalian genetic screens. Genes 6, 2781–2790 (2016).
29. Smith, R. P. et al. Massively parallel decoding of mammalian regulatory sequences supports a flexible organizational model. Nat. Genet. 45, 1021–1028 (2013).
30. GTEx Consortium. The Genotype-Tissue Expression (GTEx) pilot analysis: multitissue gene regulation in humans. Science 348, 648–660 (2015).
31. Shiraki, T. et al. Cap analysis gene expression for high-throughput analysis of transcriptional starting point and identification of promoter usage. Proc. Natl. Acad. Sci. USA 100, 15776–15781 (2003).
32. FANTOM Consortium et al. Supplementary figures, tables and texts for FANTOM 5 phase 2. Figshare https://doi.org/10.6084/m9.figshare.1288777 (2015).
33. Andersson, R. et al. An atlas of active enhancers across human cell types and tissues. Nature 507, 455–461 (2014).
34. Engreitz, J. M. et al. Local regulation of gene expression by lncRNA promoters, transcription and splicing. Nucleic Acids Res. 43, 3532–3541 (2015).
35. van Arensbergen, J. et al. Genome-wide mapping of autonomous promoter activity in human cells. Nat. Biotechnol. https://doi.org/10.1038/nbt.3754 (2016).
36. Kvon, E. Z., Stampfel, G., Yáñez-Cuna, J. O., Dickson, B. J. & Stark, A. HOT regions function as patterned developmental enhancers and have a distinct cis-regulatory signature. Genes Dev. 30, 908–913 (2016).
37. Mikhaylichenko, O. et al. The degree of enhancer or promoter activity is reflected by the levels and directionality of eRNA transcription. Genes Dev. 32, 42–57 (2018).
38. Weingarten-Gabbay, S. et al. Systematic interrogation of human promoters. Genome Res. 29, 171–183 (2019).
39. Plesa, C., Sudore, A. M., Lubock, N. B., Zhang, D. & Kosuri, S. Multiple gene synthesis in emulsions for exploring protein functional landscapes. Science 359, 343–347 (2018).

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Analysis

Methods

Design, barcoding and cloning of the enhancer library into the HSS vector.

We used an existing array library from Inoue et al. This library consists of 2,440 unique 171-bp candidate enhancer sequences, based on ChIP-seq peaks in HepG2. Each sequence was ranked with a P-value on the (Original_Array_5adapter) and a 45-bp sequence on the 3’ end (Original_Array_3adapter) (Supplementary Table 8). More detail on enhancer design can be found in that manuscript. We first amplified the library using the following primers: STARR-Seq-Ag-f and spacer-Ag-r (Supplementary Table 8). These amplify the library including the previously assigned degenerate barcodes, which align homology to the STARR-seq vector (Addgene, 71509) on the 5’ end and a spacer sequence on the 3’ end that we use for all subsequent libraries. We amplified 10 ng of array oligonucleotides with KAPA HiFi 2x Realtime (Kapa Biosystems) with a 65°C annealing temperature and 30 s extension, following the manufacturer’s protocol. We followed the reaction in real time using Sybr Green (Thermo Fisher Scientific) and stopped the reaction before plateauing, after ten cycles. We then purified the PCR product with a 1.8X AMPure XP (Beckman Coulter) cleanup and eluted in 50 µl of Qagen Elution Buffer (EB), following the manufacturer’s protocol. We took 1 µl of purified PCR product and amplified in triplicate a second reaction using KAPA HiFi 2x Realtime using primers STARR-Seq-Ag-f and STARR-BC-spacer-r with a 35 s extension time and 65°C annealing temperature for eight cycles (Supplementary Table 8). This round of PCR added a 15-bp degenerate barcode on the 3’ end of the spacer as well as homology arms to the 3’ end of the HSS vector. We then pooled the three reactions together, ran the products on a 1.5% agarose gel, and gel extracted the amplicon using the QIAquick Gel Extraction kit (Qagen), following the manufacturer’s protocol, eluting in 20 µl of Qiagen EB. We then cloned a 2:1 molar excess of our gel-extracted insert into 100 ng of the HSS vector (linearized with AgeI and Sall) with the NEBuilder HiFi DNA Assembly Cloning kit (NEB), following the manufacturer’s protocol. We transformed 10 µl of electrocompetent cells (NEB C3020) with the plasmids in duplicate following the manufacturer’s protocol, along with a no-insert negative control. We pooled the two transformations and plated on 1.5% agarose plates to estimate complexity. The following day, we estimated complexity as approximately 750,000 and grew a third of the transformation to represent a library of 250,000 in 100 ml of LB +ampicillin (Amp), so that each candidate enhancer is expected to associate on average with 100 different barcodes. We extracted the plasmid using the ZymoPURE II Plasmid Midiprep kit (Zymo Research).

Barcode association library for the nine MPRA assays. We amplified 5 ng of the HSS library with the following primers: PS5-STAR-AG-ass-f and PS7-STAR-AG-r (Supplementary Table 8). These primers add a sample-specific barcode and Illumina flow cell adapters. We then spiked the library into a NextSeq Mid 300 Cycle kit with paired-end 149-bp reads and a 20-bp index read (which captured the 15-bp barcode as well as 5 bp of extra sequence to help filter for read quality), using the following custom primers: Read1 as STAR-AG-seq-R1, Read2 as spacer-seq-R2, Index1 as PLS-mP-ssq-seq-ind1, and Index2 as STAR-AG-ind2 (Supplementary Table 8).

Library cloning. From HSS to ORI vector. We amplified 5 ng of the HSS library with the following primers: STARR-Seq-Ag-f and STARR-Seq-Ag-r (Supplementary Table 8) using KAPA HiFi 2x Realtime (Kapa Biosystems) with a 65°C annealing temperature and 30 s extension. These primers amplify both candidate enhancers and previously assigned degenerate barcodes, and add homology arms to the ORI vector (Addgene, 99296). We followed the reaction in real time with Sybr Green (Thermo Fisher Scientific) and stopped the reaction before plateauing at 13 cycles. We gel extracted the amplicon on a 1.5% agarose gel as described above. We then cloned the library in a 2:1 molar excess into 100 ng of the HSS_5adapter, ORI vector (Addgene, 99296), linearized with AgeI and Sall, with the NEBuilder HiFi DNA Assembly Cloning kit (NEB), following the manufacturer’s protocol. We then transformed 10 µl of electrocompetent cells (NEB C3020) with the plasmids in duplicate following the manufacturer’s protocol, along with a no-insert negative control. We pooled the two transformations and plated on 1.5% agarose plates to estimate complexity. The following day, we estimated the complexity as >500,000 and extracted the plasmid using the ZymoPURE II Plasmid Midiprep kit (Zymo Research).

From HSS to PGL4.23c MPRA vector. As described above, we amplified 5 ng of the HSS library with the following primers: PGL4.23c-AG-1f and PGL4.23c-AG-1r (Supplementary Table 8). These primers amplify both candidate enhancers and previously assigned degenerate barcodes, and add homology arms to the PGL4.23c MPRA vector (GenBank MK484105). We stopped the reaction before plateauing at 18 cycles. We linearized the pGL4.23c MPRA backbone, while removing the minimal promoter, using KpnI and XbaI. We then cloned the linearized plasmid with an X-tremeGENE:DNA ratio of 2:1. We cloned the insert into the plasmid backbone (Addgene, 81225), which had been linearized with AgeI and EcoRI only and treated with Antarctic Phosphatase. We used similar methods as in the pGL4.23c library cloning with the following changes. The HSS library was amplified with pLS-mP-AG-2f and pLS-mP-AG-5r (Supplementary Table 8) for 17 cycles. After gel extraction, we cloned the insert into the pLS-mP backbone (Addgene, 81225), which had been linearized with AgeI and EcoRI only and treated with Antarctic Phosphatase.

Cell culture, lentivirus packaging and titration. HEK293T and HepG2 cell culture, lentivirus packaging and titration were performed as previously described with modifications. Briefly, 12 million HEK293T cells were seeded in 15 cm dishes and cultured for 48 h. To generate WT lentiviral libraries (5'/5'T, 5'/3'T and 3'/3'T WT), the cells were co-transfected with 5.5 µg of pMMPRA libraries, 1.85 µg of pMD2.G (Addgene, 12259) and 3.65 µg of psPAX2 (Addgene, 12280), which encodes a WT pol, using EndoFectin Lentivector transfection reagent (GeneCopoeia) according to the manufacturer’s instruction. To generate nonintegrating lentiviral libraries (5'/5'T, 5'/3'T and 3'/3'T MT), pLV-Help (InvivoGen) that encodes a mutant pol was used instead of psPAX2. After 18 h, cell culture medium was refreshed and TiterBoost reagent (GeneCopoeia) was added. The transfected cells were cultured for 2 d and lentivirus collected and concentrated using the Lenti-X concentrator (Takara) according to the manufacturer’s protocol. To measure UMI DNA signal for the lentivirus libraries, HepG2 cells were plated at 1 x 10⁶ cells per well in 24-well plates and incubated for 24h. Serial volume (0, 4, 8 and 16 µl) of the lentiviral was added with 8 µg ml⁻¹ polybrene to increase infection efficiency. The infected cells were cultured for 3 d and then washed with PBS three times. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). A gel was cut at 500 bp and the mean was measured as relative amount of viral DNA (WPRE region, WPRE_F and WPRE_F over that of genomic DNA (intronic region of LIPC gene, LIPC_F and LIPC_R, Supplementary Table 8) by qPCR using SsoFast EvaGreen Supermix (Bio-Rad), according to the manufacturer’s protocol.
manufacturer's instructions and eluted in a total of 344 µl of Qiagen EB. The entire purified product was then used for a second round of PCR, split into 16 × 50 µl reactions each, with primers P5 and P7 (Supplementary Table 8). The reaction was followed in real time with Sybr Green (Thermo Fisher Scientific) and fragment A included positions 1–190 and fragment B included positions 161–354. To fragment A, we appended the HSSF-ATGC adapter to the 5’ end and the HSS-R-clon adapter to the 3’ end (Supplementary Table 8).

For the 354-bp library, we split each sequence into two overlapping fragments, A and B. Fragment A included positions 1–190 and fragment B included positions 161–354. To fragment A, we appended the HSSF-ATGC adapter to the 5’ end and the HSS-R-clon adapter to the 3’ end (Supplementary Table 8). For the 678-bp library, we only designed the 2,236 sequences from Inoue et al.14. We split the sequences into 13 different sets of 172 sequences each. We then split each sequence into two new fragments. Fragment A included positions 1–190, fragment B included positions 161–352, fragment C included positions 323–514 and fragment D included positions 485–678. Adapters and primers used for the 13 sets of HMPS are included in Supplementary Table 10.

**Amplification of the 192-bp library.** All 192-bp enhancers were amplified from the array using HSSF-ATGC and HSS-R-clon (Supplementary Table 8) with KAPA HiFi HotStart Uracil+ ReadyMix PCR kit (Kapa Biosystems) with Sybr Green (Thermo Fisher Scientific) on a MiniOpticon Real-Time PCR System (Bio-Rad) and stopped before plateauing.

**Multiplex pairwise assembly for 354-bp library.** All 5’ fragments were amplified off the array using HSSF-ATGC and DO_15R_PU (Supplementary Table 8) with KAPA HiFi HotStart Uracil+ ReadyMix PCR kit (Kapa Biosystems) and stopped before plateauing. All 3’ fragments were amplified off the array using DO_3F_PU and HSS-95R (Supplementary Table 8). Both were purified with a 1.8X AMPure cleanup and eluted in 20 µl of USER enzyme (Thermo Fisher Scientific) and then 2 µl of USER enzyme (NEB) was added directly to each purified PCR product and incubated for 15 min at 37 °C followed by 15 min at room temperature. Reactions were then treated with the NEBNext End Repair Module (NEB) following the manufacturer's protocol and purified using the DNA Clean and Concentrator 5 (Zymo Research) and eluted in 12 µl EB, following the manufacturer's protocol. We then quantified DNA concentrations using a Qubit and purified DNA to 0.75 ng/µl. We then assembled the 5’ and 3’ fragments as described previously14. Briefly, fragments were allowed to anneal and extend for five cycles with KAPA HiFi 2x HotStart Readymix (Kapa Biosystems) before primers HSSF-ATGC and DO_95R were added for amplification (Supplementary Table 8).

**Hierarchical multiplex pairwise assembly for 678-bp library.** All libraries were amplified off the array using the primers indicated in Supplementary Table 10 with KAPA HiFi HotStart Uracil+ ReadyMix PCR Kit (Kapa Biosystems) as described above. During the first round of assembly, fragments A and B were assembled using HSSF-ATGC and DO_3F_PU and fragments C and D were assembled using DO_8E_PU and HSS_R-clon for the reverse orientation, 20 ng of the backbone was linearized with Len_lib_linF and Len_lib_linR (Supplementary Table 8). We then assembled the 5’ and 3’ fragments as described previously14. Briefly, fragments were allowed to anneal and extend for five cycles with KAPA HiFi 2x HotStart Readymix (Kapa Biosystems) before primers HSSF-ATGC and DO_95R were added for amplification (Supplementary Table 8).

**Sequence validation of assembled libraries.** Before cloning, we verified assembly and uniformity of our libraries. The multiplex pairwise assembly library (2,336 354-mers) was sequenced on a Miseq v.3 600 cycle kit with paired-end 305 bp reads. Reads were merged with PEAR v0.9.5 (ref.15) and aligned to a reference fasta file with BWA mem v0.7.10–789 (ref.16). Each of the 13 hierarchical pairwise assembly libraries included 192 enhancer-length fragments as well as different combinations of sets, up to all 2,236 sequences at once41. Assemblies AB and CD were then assembled together following the multiplex pairwise assembly protocol42. After the second assembly, all embedded DNA was purified with a 0.6X AMPure cleanup and eluted in 30 µl EB. We then amplified 1 µl of each assembly with HSSF-ATGC-puF and HSS-R-clon-puR1 to add flow cell adaptors and indexes (Supplementary Table 8). We performed the assembly for each set of 172 sequences separately, as well as for different combinations of sets, up to all 2,236 sequences at once14.
Analysis

High-Fidelity 2x PCR Master Mix (NEB) for 16 cycles. For the 678-mer libraries, we pooled all 13 sub-libraries at equal concentrations and then amplified 20 ng of the same primers and conditions above. All PCR products were purified with 1× AMPure cleanup following the manufacturer's instructions and eluted in 50 µl. We then used 1 µl of each purified reaction for a second PCR to amplify the 15-bp degenerate barcodes and cloning adaptors. For the second reaction, we used HSS-pGL4_F and HSS_pGL4_R2 (Supplementary Table 8).

We linearized the pGL4.23c MPRA backbone, while removing the minimal promoter and reporter using HindIII and XhoI. We treated the linearized plasmid with Antarctic Phosphatase following the manufacturer's protocol and then gel extracted the plasmid on a 1% agarose gel. We then cloned all three libraries into the pGL4.23c plasmid using the NEBuilder HiFi DNA Assembly Cloning kit (NEB), following the manufacturer's protocol. At 48 h after transfection, we extracted DNA and RNA from each replicate using the AllPrep DNA/RNA Mini Kit (Qiagen), following the manufacturer's instructions.

For each of the barcode association reactions, we used NEBuilder HiFi DNA Assembly Cloning kit (NEB) as described above.

MPRA of all enhancer-length libraries. The day before transfection, we seeded HepG2 cells in five 10-cm dishes. On the day of transfection, we combined the 25, 192, 354 and 678 mers of each library at a 1:1 ratio in 100 µl of the 1× AMPure cleanup following the manufacturer's instructions and eluted in 50 µl. We then used 2.5 µl of each library for transfection with 2 µg of pGL4.23c-U6 (Promega). We then treated the linearized plasmid with XbaI and BglII and its concentration was adjusted to 15 ng/µl. We then linearized the plasmid with XbaI and BglII and cloned it into the pGL4.23c-U6 promoter using NEBuilder HiFi DNA Assembly Cloning kit (NEB). We then inserted the minimal promoter and GFP using the NEBuilder HiFi DNA Assembly Cloning kit (NEB) as described above.

For each of the 1143 DNA samples for each MPRA experiment, we used UMI's to collapse barcodes derived from the same molecule during PCR and mapped to the element they were linked to, as identified by the dictionary of barcode–element associations. To normalize RNA and DNA for different sequencing depths in each sample, we followed a nearly identical scheme as one we had previously devised.

Briefly, for each replicate of each MPRA design, we first considered the subset of barcodes that were observed in both RNA and DNA samples of the replicate. We then summed up the counts of all barcodes contributing to each element and computed the normalized counts as the counts per million (cpm) sequenced reads of that library. Finally, we computed enhancer activity scores as log[(RNA cpm)/(DNA cpm)]. To account for the differential scale among replicates of each experiment, we divided the RNA barcode associations by their value before averaging them. Due to low counts in the initial round of sequencing and poor sample quality, the three replicates from the 5′/3′ MT and 3′/3′ MT were re-sequenced and the data from each pair of technical replicates were pooled across the two independent sequencing runs. Even after pooling, the first replicate from each of the three original technical replicates had a higher correlation and transcriptional variance than the other replicates (Fig. 2s and Supplementary Fig. 3) and thus were excluded during replicate averaging (Supplementary Table 2). In practice, this decision very modestly altered the numerical results and did not change the study's conclusions.

Modeling and analyses. Feature pre-processing. Right-skewed data such as ChIP-seq and CAGE signal were log-transformed to approximate a normal distribution, and each feature was then z score normalized to scale the features similarly. This enabled a direct comparison of coefficients among features derived from the resulting linear models.

Model training. As described before, we trained a lasso regression model on each of ten folds of the data, selecting enhancers that were measured with at least ten independent barcodes to reduce the impact of measurement noise in the assay. The model was trained with a single parameter, the regularization parameter. Specifically, because it employs an L1 regularization penalty, which leads to the selection of the fewest features that maximally explain the data. The strength of the regularization was controlled by a single λ parameter, which was optimized using tenfold CV on the entire dataset. To evaluate the most relevant features selected, we trained a lasso regression model on the full dataset and visualized the top 10–30 coefficients with the maximum magnitude. A full table of the selected features and their coefficients are provided (Supplementary Table 5). To compare differential enhancer activity between a pair of assays, we fit a loss (locally estimated scatterplot smoothing) regression between one assay relative to the other and computed residuals from this fit, using the ‘loss’ function in R. We then fitted lasso regression models to explain these residuals, based upon the aforementioned procedure.

Luciferase assays. The 'medium', 'long' and 'deleted' versions of ten enhancers (total 30 sequences), APOE enhancer (positive control) and neg2 sequence (negative control) were synthesized along with minimal promoter and adaptor sequences (Supplementary Table 8) and cloned into the BglII and Ncol site of the pGL4.23c vector by Twist Bioscience. These were selected on the basis of highest differential activities, reproducibility and base balance (for synthesis). As two of them (chr2:106744003-106744357, medium and chr10:114391246-114391924, del) failed the cloning, these sequences were synthesized by Twist Bioscience and manually cloned into the BglII and Ncol site of the pGL4.23c vector by Twist Bioscience. These were selected on the basis of highest differential activities, reproducibility and base balance (for synthesis). All 32 plasmids and empty pGL4.23c were individually transfected along with pGL4.74 (Promega) into 1×10⁶ HepG2 cells, as previously described.

Four independent replicate cultures were transfected. Firefly and Renilla luciferase activities were measured on a Glomax microplate reader (Promega) using the Dual-Luciferase Reporter Assay System (Promega).
Enhancer activity was measured as the fold change of each plasmid's firefly luciferase activity normalized to Renilla luciferase activity.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
We developed a fully reproducible MPRA processing pipeline available to process the data into final enhancer activity scores. Raw and processed data have been deposited in the Gene Expression Omnibus at accession number GSE142696.

**Code availability**
A reproducible processing pipeline for MPRA data is available as a Nextflow-based MPRA processing pipeline named MPRAflow (https://github.com/shendurelab/MPRAflow)*.

**References**
40. Quinlan, A. R. & Hall, I. M. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26**, 841–842 (2010).
41. Klein J. C. et al. A systematic evaluation of the design, orientation, and sequence context dependencies of massively parallel reporter assays. *Protoc. Exch.* https://doi.org/10.21203/rs.3.pex-1065/v1 (2020).
42. Zhang, J., Kobert, K., Flouri, T. & Stamatakis, A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* **30**, 614–620 (2014).
43. Li, H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. Preprint at arXiv https://arxiv.org/abs/1303.3997 (2013).
44. Gordon, M. G. et al. lentiMPRA and MPRAflow for high-throughput functional characterization of gene regulatory elements. *Nat. Protoc.* **15**, 2387–2412 (2020).
45. Karolchik, D. et al. The UCSC Genome Browser database: 2014 update. *Nucleic Acids Res.* **42**, D764–D770 (2014).
46. Zhou, J. & Troyanskaya, O. G. Predicting effects of noncoding variants with deep learning-based sequence model. *Nat. Methods* **12**, 931–934 (2015).
47. Alipanahi, B., Delong, A., Wei, H., B. J. Predicting the sequence specificities of DNA- and RNA-binding proteins by deep learning. *Nat. Biotechnol.* **33**, 831–838 (2015).
48. Roadmap Epigenomics Consortium. et al. Integrative analysis of 111 reference human epigenomes. *Nature* **518**, 317–330 (2015).

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**Author contributions**
J.K. and A.K. performed all cloning and sequencing for the nine assays and all experimental work for orientation and length sections. J.K. and J.S. conceived the HMPA protocol, and J.K. and A.K. developed and optimized it. A.K. produced schematic figures. M.K. developed the initial MPRA analysis pipeline. V.A. performed the computational analyses and generated all remaining figures and tables. F.I. performed the transfections and lentiviral transductions for the nine assays, carried out luciferase reporter experiments and wrote the associated methods sections. B.M. designed cloning steps and guided the development and testing of the MPRA assays. J.K., V.A., N.A. and J.S. wrote the remainder of the paper. N.A. and J.S. supervised the project.

**Competing interests**
V.A. is an employee of Calico Life Sciences LLC.

**Additional information**
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*Correspondence and requests for materials* should be addressed to N.A. or J.S.

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**Software and code**

Policy information about availability of computer code

Data collection: All software used for data processing are noted in the Methods section and appropriately cited. They are: PEAR v0.9.5, BWA mem v0.7.10-r789, bc12fastq v2.18, DeepSEA v0.94, DeepBind v0.11, MPRAflow

Data analysis: The full MPRA analysis pipeline in this study can be found at http://github.com/shendurelab/MPRAflow

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| Sample size | Sample sizes are noted in each of the figures and resulted from the number of elements designed that were successfully measured after using our processing pipeline |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | Two technical replicates (replicate 1 of 5/3MT and 3/3MT) were excluded during averaging due to poor sample quality, and these were directly noted in our Methods sections and text. |
| Replication | Each of the assays were performed with either three or four replicates to ensure faithful reproduction of the assay. All attempts at replication were successful. |
| Randomization | Not relevant, as we needed to know the identity of each sample prior to their analyses as each design backbone and therefore initial steps in processing are unique. |
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Eukaryotic cell lines

Policy information about cell lines

| Cell line source(s) | HepG2 cells (ATCC HB-8065), HEK203T (ATCC-CRL-3216) |
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| Authentication | HepG2 cells and Hek293T cells were not authenticated |
| Mycoplasma contamination | HepG2 cells and Hek293T cells were not tested for mycoplasma contamination |
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