the appropriate EvoD version can be applied. Whether the method was properly trained and tested within each conservation stratum is a separate question. Vihinen[1] correctly notes that some of the procedural details surrounding EvoD were lacking, prompting Kumar *et al.*[2] to provide a more thorough description in their response. The additional information supports that EvoD was sensibly calibrated and evaluated.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

COMPETING FINANCIAL INTERESTS
The author declares no competing financial interests.

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1. Vihinen, M. *Nat. Methods* **11**, 781 (2014).
2. Kumar, S., Ye, J. & Liu, L. *Nat. Methods* **11**, 781–782 (2014).
3. Kumar, S. *et al. Nat. Methods* **9**, 855-856 (2012).
4. Eiland, E.E. & Liebrock, L.M. *Adv. Artificial Intelligence* **2013**, 42795 (2013).

Improved vectors and genome-wide libraries for CRISPR screening

To the Editor: Genome-wide, targeted loss-of-function pooled screens using the clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated nuclease Cas9 in human and mouse cells provide an alternative screening system to RNA interference (RNAi)[1–4]. Previously, we used a genome-scale CRISPR knockout (GeCKO) library to identify loss-of-function mutations conferring vemurafenib resistance in a melanoma model[1]. However, initial lentiviral delivery systems for CRISPR screening had low viral titer or required a cell line already expressing Cas9, thereby limiting the range of biological systems amenable to screening.

We sought to improve both the lentiviral packaging and choice of guide sequences in our original GeCKO library[1], where a pooled library of synthesized oligonucleotides was cloned into a lentiviral backbone containing both the *Streptococcus pyogenes* Cas9 nuclease and the single guide RNA (sgRNA) scaffold. To create a new vector capable of producing higher-titer virus (lentiCRISPRv2), we made several modifications, including removal of one of the nuclear localization signals, human-codon optimization of the remaining nuclear localization signal and P2A bicistronic linker sequences, and repositioning of the U6-driven sgRNA cassette (**Fig. 1a**). These changes resulted in an approximately tenfold increase in functional viral titer over that of lentiCRISPRv1 (ref. 1; **Fig. 1b**).

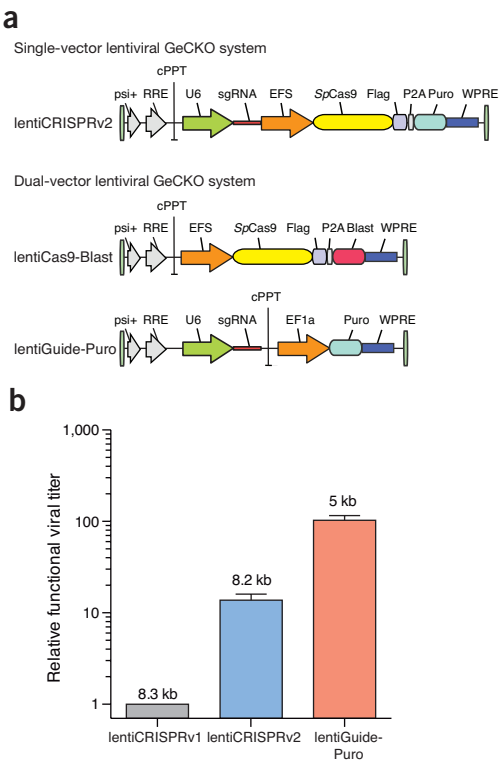

Figure 1 | CRISPR lentiviral vectors with higher functional titer. (**a**) Lentiviral expression vector for *S. pyogenes* Cas9 (*Sp*Cas9) and sgRNA in the improved one-vector system (lentiCRISPRv2) and the two-vector system (lentiCas9-Blast, lentiGuide-Puro). psi+, Psi packaging signal; RRE, Rev response element; cPPT, central polypurine tract; EFS, elongation factor 1α short promoter; Flag, Flag octapeptide tag; P2A, 2A self-cleaving peptide; Puro, puromycin selection marker; WPRE, post-transcriptional regulatory element; Blast, blasticidin selection marker; EF1a, elongation factor 1a promoter. (**b**) Relative functional titer of viruses made from the indicated vectors, with an EGFP-targeting sgRNA (mean ± s.e.m., $n = 3$ independently transfected virus batches with 3 replicate transductions into HEK293FT cells per construct). Numbers above each bar indicate the size of the packaged virus for each construct.

Table 1 | Comparison of GeCKOv2 sgRNA libraries with existing CRISPR libraries

	Wang *et al.*[2] library	Shalem *et al.*[1] GeCKOv1 library	Koike-Yusa *et al.*[3] library	GeCKOv2 human library	GeCKOv2 mouse library
Species	Human	Human	Mouse	Human	Mouse
Genes targeted	7,114	18,080	19,150	19,050	20,611
Targeting constructs per gene	10	Variable (typically 3 or 4)	Variable (typically 4 or 5)	6	6
miRNAs targeted	None	None	None	1,864	1,175
Targeting constructs per miRNA	N/A	N/A	N/A	4	4
Control (nontargeting) sgRNAs	100	None	None	1,000	1,000
Total sgRNA constructs	73,151	64,751	87,897	123,411	130,209
Viral plasmid vector	Dual vector: sgRNA only	Single vector: Cas9 and sgRNA (lentiCRISPRv1)	Dual vector: sgRNA only	Single and dual vector: lentiCRISPRv2 and lentiGuide-Puro	Single and dual vector: lentiCRISPRv2 and lentiGuide-Puro

To further increase viral titer, we also cloned a two-vector system, in which Cas9 (lentiCas9-Blast) and sgRNA (lentiGuide-Puro) are delivered using separate viral vectors with distinct antibiotic selection markers (**Fig. 1a**). lentiGuide-Puro had an ~100-fold increase in functional viral titer over that of the original lentiCRISPRv1 (**Fig. 1b**). Both the single- and dual-vector systems mediated efficient knockout of a genomically integrated copy of EGFP in human cells (**Supplementary Fig. 1**). Whereas the dual-vector system enables generation of Cas9-expressing cell lines that can be subsequently used for screens using lentiGuide-Puro, the single-vector lentiCRISPRv2 may be better suited for *in vivo* or primary-cell screening applications.

We also designed and synthesized new human and mouse GeCKOv2 sgRNA libraries (**Supplementary Methods**) with several improvements (**Table 1**). First, for both human and mouse libraries, to target all genes with a uniform number of sgRNAs, we selected six sgRNAs per gene distributed over three or four constitutively expressed exons. Second, to further minimize off-target genome modification, we improved the calculation of off-target scores on the basis of specificity analysis[5]. Third, to inactivate microRNAs (miRNAs), which play a key role in transcriptional regulation, we added sgRNAs that direct mutations to the pre-miRNA hairpin structure[6]. Finally, we targeted ~1,000 genes not included in the original GeCKO library.

Each library, mouse and human, is divided into two sublibraries, each containing three sgRNAs targeting every gene as well as 1,000 nontargeting control sgRNAs. Screens can be performed by combining both sublibraries, yielding six sgRNAs per gene. Alternatively, individual sublibraries can be used in situations in which cell numbers are limiting (for example, with primary cells or *in vivo* screens). We cloned both human and mouse libraries into lentiCRISPRv2 and lentiGuide-Puro and sequenced them to ensure uniform representation (**Supplementary Figs. 2** and **3**). These new lentiviral vectors (see **Supplementary Data** for full sequences) and libraries further expand the GeCKO toolbox for diverse screening applications. Reagents are available to the academic community through Addgene (lentiCRISPRv2: 52961; lentiCas9-Blast: 52962; lentiGuide-Puro: 52963; human GeCKOv2 in lentiCRISPRv2: 1000000048; human GeCKOv2 in lentiGuide-Puro: 1000000049; mouse GeCKOv2 in lentiCRISPRv2: 1000000052; mouse GeCKOv2 in lentiGuide-Puro: 1000000053). Associated protocols, support forums and computational tools are available at http://www.genome-engineering.org/.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper (doi:10.1038/nmeth.3047).

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AUTHOR CONTRIBUTIONS

N.E.S., O.S. and F.Z. conceived of and designed the experiments. N.E.S. and O.S. performed the experiments and analyzed the data. N.S., O.S. and F.Z. wrote the manuscript.

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1. Shalem, O. *et al. Science* **343**, 84–87 (2014).
2. Wang, T., Wei, J.J., Sabatini, D.M. & Lander, E.S. *Science* **343**, 80–84 (2014).
3. Koike-Yusa, H., Li, Y., Tan, E.-P., Velasco-Herrera, M.D.C. & Yusa, K. *Nat. Biotechnol.* **32**, 267–273 (2014).
4. Zhou, Y. *et al. Nature* **509**, 487–491 (2014).
5. Hsu, P.D. *et al. Nat. Biotechnol.* **31**, 827–832 (2013).
6. Zhao, Y. *et al. Sci. Rep.* **4**, 3943 (2014).

iPipet: sample handling using a tablet

To the Editor: Biological experiments increasingly involve large numbers of specimens, making liquid handling in these experiments a challenge. We and other groups previously devised high-throughput experimental designs using combinatorial pooling schemes that reduce experiment costs but require complex pipetting steps according to mathematical patterns[1–3]. We used a liquid-handling robot to execute experiments with bacteria[4], but we found that using a robot with sensitive human samples has several caveats and inherent limitations, such as occasional robotic failures, dead volume (inability to aspirate liquid close to the bottom of the well) and bending or clogging of tips owing to plate septum piercing that risked finite samples. In addition, liquid-handling robots are quite expensive and require trained personnel to operate them.

Several devices offer semi-automated solutions for pipetting complex protocols that mainly consist of a programmable LED panel with lights under the wells of microtiter plates that guide pipetting (**Supplementary Table 1**). But these devices support a relatively narrow set of designs, have minimal visual cues and do not display volumes. In addition, their price range is about $1,000–$2,000.

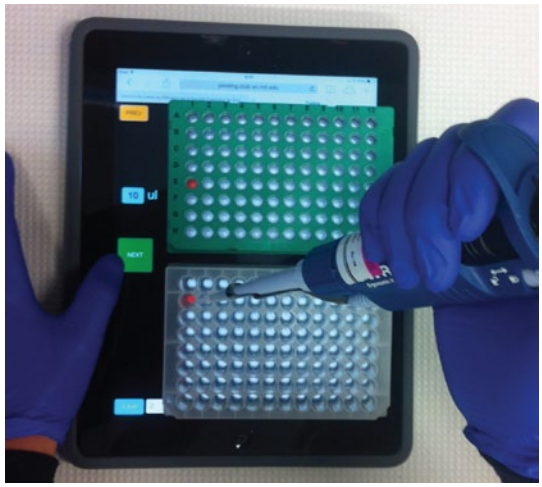

Figure 1 | A bird's-eye view of the iPipet run screen with 96-well plates.