TITLE

Prevalence of errors in lab-made plasmids across the globe

AUTHORS

Xingjian Bai[1], Jack F. Hong[1], Shan Yu[1], David Y. Hu[1], Amy Y. Chen[1], Constance A. Rich[1], Silk J. Shi[1], Sandy Y. Xu[1], Daniel M. Croucher[1], Kristofer J. Müssar[1], Daniel W. Meng[1], Jane L. Chen[1] and Bruce T. Lahn[1,2,3]

AFFILIATIONS

[1]VectorBuilder, Chicago, IL 60609, USA

[2]Future Institute of Gene Delivery Research, Guangzhou, China

[3]To whom correspondence should be addressed: brucelahn@vectorbuilder.net

ABSTRACT

Plasmids are indispensable in life sciences research and therapeutics development. Currently, most labs custom-build their plasmids. As yet, no systematic data on the quality of lab-made plasmids exist. Here, we report a broad survey of plasmids from hundreds of academic and industrial labs worldwide. We show that nearly half of them contained design and/or sequence errors. For transfer plasmids used in making AAV vectors, which are widely used in gene therapy, about 40% carried mutations in ITR regions due to their inherent instability, which is influenced by flanking GC content. We also list genes difficult to clone into plasmid or package into virus due to their toxicity. Our finding raises serious concerns over the trustworthiness of lab-made plasmids, which parallels the underappreciated mycoplasma contamination and misidentified mammalian cell lines reported previously, and highlights the need for community-wide standards to uphold the quality of this ubiquitous reagent in research and medicine.

INTRODUCTION

Plasmids are extrachromosomal DNA capable of independent replication in cells. They are most commonly found in bacteria in circular double-stranded form. Plasmids were first identified in bacterial antibiotic resistance studies in the 1950s[1]. In the 1970s, recombinant DNA technology enabled the engineering of artificial plasmids carrying foreign DNA of interest[2], which in the ensuing years propelled plasmids to become a central and ubiquitous reagent in the life sciences. Nowadays, plasmids are used mostly as gene delivery vectors in vitro and in vivo, either directly or as starting materials for generating viral and mRNA vectors. In addition to their ubiquity in research, plasmids have also become a foundational source material in manufacturing many therapeutic products such as recombinant protein drugs including antibodies, gene therapy vectors, and the recent Covid-19 mRNA vaccines.

Plasmids are a highly customized reagent because different experimental applications generally require different plasmids. For many decades, researchers have typically constructed their own plasmids in the lab or shared them from other researchers. As yet, there is no systematic quality assessment of lab-made plasmids on a global scale despite their importance in research and medicine, likely because such an endeavor would be impractical for any single lab.

As a cloning service provider, we received a large number of lab-made plasmids along with their theoretical sequences from both academia and industry across the world, which accorded us an opportunity to systematically assess their quality. We observed a wide variety of design errors ranging from obvious ones that most trained molecular biologists can identify, to subtle mistakes that even very seasoned experts may not spot. Sequence errors are even more prevalent. In combination, design and sequence errors affect nearly half of the lab-made plasmids we received.

We paid special attention to AAV transfer plasmids used to package recombinant AAV virus because they were often used to develop gene therapy drugs. We found that their ITRs were highly mutable, with about 40% of the plasmids we received bearing mutations relative to wildtype ITR sequence. We further showed that ITR instability is associated with high GC content of the immediate flanking sequence.

Researchers typically send plasmids to us for further sequence modification, recombinant viral vector production, and/or in vitro and in vivo experiments. Given that the senders are devoting significant financial resources and time to contract us to perform these downstream projects, they have a vested interest in ensuring the correctness of their plasmids. Considering this, it is possible that the quality issues we uncovered might underestimate the true scale of the problems in labs. Similar to the reports of mycoplasma contamination and misidentified mammalian cell lines[3-5], our comprehensive survey shines a spotlight on significant quality issues with lab-made and shared plasmids in academia and industry around the world.

RESULTS

Prevalence of design errors in lab-made plasmids

When receiving plasmids from researchers to perform various projects such as cloning, virus packaging and IVT RNA production, we would first evaluate the reference maps and sequences provided by the senders to ensure that the vectors were appropriately designed. Strikingly, of 2,521 plasmids received from academia and industry around the world that we included in this survey, about 15% (384) were found to contain significant design errors that could impact function. These errors were found in most types of components, with the most prevalent being the incorrect choice or placements of promoters, followed by problems in the choice or design of

open reading frames (ORFs). Details of common design errors and their corresponding consequences and frequencies are listed in Table 1.

Many errors appear to be due to insufficient understanding of the nuances in designing appropriate gene delivery systems, such as limits on cargo capacity, promoter silencing, and considerations for different types of linkers (Table 1). Other problems are related to specific sequence characteristics of individual vectors. Some sequences are unstable in E. coli, such as long inverted repeats that are prone to form large hairpins, extremely high-GC sequences, and short tandem repeats (e.g. long strings of mono-, di-, or tri-nucleotides, including A tracts in template plasmids for making IVT RNA). When these sequences are cloned into high-copy plasmids, they can quickly accumulate mutations, including large deletions and rearrangements (Table 1). For greater stability, it is necessary to clone these sequences into low-copy plasmids along with tailored E. coli hosts, and utilize special culture conditions such as low temperature, low salt, and adjusted antibiotic concentration. The sequences themselves can often be modified to increase stability while maintaining biological functions, e.g. placing a short intervening sequence in the long A tract in in vitro transcription plasmids.

Additionally, a major issue that sometimes plagues lab-made plasmids is toxicity of the gene of interest (GOI) that they carry. If the GOI is toxic to E. coli, then cloning it into a plasmid can be very difficult and sometimes impossible. In cases where cloning is successful, the GOI or its surrounding sequences tend to be highly unstable and can quickly accumulate mutations that compromise GOI function (unpublished data), presumably due to strong selective pressure against the intact toxic form of the gene. By design, there are typically no prokaryotic promoters driving expression of the toxic GOIs, so the fact that they can still exert their detrimental effect on the host indicates the presence of cryptic promoters driving their expression in E. coli. Similarly, genes contained in viral transfer plasmids can be toxic to packaging cells or interfere with virus packaging pathways, leading to dramatically reduced packaging efficiency and viral titer. Unfortunately, toxicity of genes is often hard to predict even for labs working with them.

Table S1 and S2 list genes showing toxicity to E. coli host that we have encountered. Table S1 contains 46 genes that are moderately toxic, and their cloning in intact forms can often be accomplished by employing various workarounds such as using low-copy plasmids, switching to different E. coli host strains, and altering culture conditions. Table S2 lists 25 genes that are severely toxic, and their cloning in intact forms was unsuccessful in our hands by the above workarounds alone, though we managed to clone most of them in various mutated forms such as introducing point mutations or truncations, and inserting synthetic introns. These toxic genes are enriched for membrane channels and transporters, and proteins involved in DNA dynamics such as DNA repair, topoisomerase activity, and chromosome segregation. Particularly striking is the enrichment for calcium and sodium channels, with each type accounting for about ten (14%) of the toxic genes listed. There are also two chloride channels on the list. This enrichment is presumably due to these channel genes causing ion imbalances in E. coli host. Indeed, the toxicity of ion channel genes in cloning may be a rule rather than an exception.

Table S3 lists 73 genes that we found to be toxic to virus packaging, resulting in very low viral titer in at least some cases. They are enriched for pro-apoptotic genes (e.g. BAX and N-GSDME), cell cycle regulators (e.g. BABAM2 and NEK1), proliferation modulators (e.g. F2RL1 and Foxn1), and antiviral genes (e.g. EIF2AK2 and APOBEC3A). Interestingly, a gene that severely inhibits packaging efficiency when placed in a particular viral transfer plasmid may not have the same detrimental effect when placed in another transfer plasmid, presumably because its toxic effect also depends on other factors such as the strength of the promoter driving the GOI in packaging cells, the type of virus being packaged (e.g. lentivirus vs. AAV), and the packaging cell lines used. One solution often effective in reducing GOI toxicity to virus packaging is to use weaker or inducible promoters. For example, a lentiviral vector containing a

medium-strength promoter driving mouse Foxn1 produced tenfold higher titer as compared to the same vector using a strong promoter.

Prevalence of sequence errors in lab-made plasmids

We subjected 1132 plasmids provided by researchers to further QC validation. Of these, about 1.9% (21/1132) could not be recovered from the E. coli stocks we received or the incorrect samples were sent. We analyzed the overall structure of 852 plasmids by restriction enzyme (RE) digestion, selecting multiple RE sites from the sender-provided vector maps and sequences that were expected to yield distinct fragments upon digestion. The other plasmids were sequenced directly without RE digestion. Remarkably, RE digestion of 852 plasmids revealed inconsistent fragment patterns in about 15% (128/852), indicating significant rearrangements of these plasmids or point mutations at the RE sites (Figure 1A). Given that RE digestion only confirmed the general structure of the plasmids, we also performed sequencing-based validation on some plasmids, focusing on functional regions utilized in downstream cloning or crucial for intended biological applications. Here, ITR regions of AAV transfer plasmids were excluded from analysis because their sequence mutations were evaluated separately (see below for detailed description). We Sanger sequenced 117 plasmids with correct RE digestion patterns and found that about 24% (28/117) exhibited inconsistent sequences compared to the senders' reference, and two failed to have their functional regions fully sequenced, presumably due to the presence of difficult sequences (Figure 1A). To remove any bias, we directly sequenced 259 plasmids without performing initial RE digestion, focusing on functional regions (again, excluding AAV ITRs). Notably, about 35% of these plasmids (91/259) displayed sequence variations from the senders' reference (Figure 1B). Among them, we identified 89 point mutations, 35 deletions, and 19 insertions, with some plasmids containing multiple types of errors.

ITRs of AAV transfer plasmids are highly mutable

We paid special attention to AAV vectors given their therapeutic importance[6,7] A superior feature of AAV is that the only cis sequence elements required for packaging recombinant virus are the two ITRs flanking the payload sequence in the transfer plasmid (Figure 2A, 2B). By convention, ITRs of AAV serotype 2 (AAV2) are widely utilized in recombinant AAV vectors due to their compatibility with a wide range of capsid types[7-9]. However, AAV2 ITRs contain over 70% GC and can form complex secondary structure. As a result, ITR sequences on AAV transfer plasmids can acquire mutations that impair packaging, leading to decreased full capsid ratio and increased encapsulation of cellular DNA, problems that can significantly compromise the use of recombinant AAV as a therapeutic agent[10,11].

The wildtype AAV2 viral genome is single-stranded DNA of ~4.7 kb, and can exist as either the sense or antisense strand relative to the direction of the encoded genes Rep and Cap (Figure 2A). The two ITRs that bookend the AAV2 viral genome reverse complement each other (namely, one ITR in an AAV2 genome is identical in sequence to the reverse complement of the other ITR). Each ITR is 145 nucleotides (nt) long that includes a 125-nt self-annealing sequence forming a T-shaped hairpin with two arms denoted B-B' and C-C', and a stem A-A', as well as a 20-nt single-stranded D region that extends from the hairpin (Figure 2C, 2D). The A-A' stem of ITR contains the Rep-binding element (RBE) and the terminal resolution site (trs). RBE is necessary for recruiting the Rep proteins that replicate the viral genome, while trs is used as the replication initiation site[12]. There is also a secondary Rep-binding element (RBE') on the C-C' arm that contributes to Rep recruitment[13]. The relative positioning of B-B', C-C', and A-A' regions

determines whether the configuration of an AAV2 viral genome's ITRs is "flip" (Figure 2C) or "flop" (Figure 2D), with the former having the B-B' region, while latter having C-C', closest to the open end of the AAV2 genome. For either flip or flop configuration, the ITR can exist as the strand with a 5' open end or the strand with a 3' open end. For simplicity, only the latter forms are depicted in detail in Figure 2.

The ITRs in AAV transfer plasmids almost all correspond to the flop configuration. We noticed three versions. One is a 145-bp sequence that is the same as the flop version of the full-length ITR sequence in the AAV2 viral genome as depicted in Figure 2D. But this version is very rarely used, with just a few examples out of the hundreds of AAV plasmids that we came across. The second version, which is the most prevalent, is a 130-bp sequence that corresponds to the first version except missing 15 bp at the end of the A region (Figure 2E). When transfer plasmids carrying this ITR are packaged into virus, the missing sequence is added back to form the complete viral genome ITR by copying from the A' region. Transfer plasmids for which both ITRs are the 145-bp or 130-bp version can generate comparable amounts of AAV viral particles with similar transduction capability[11]. These two versions are therefore referred as the wildtype, with one being full-length and the other partial. The third version is a 119-bp sequence that corresponds to the second version but with an additional 11-bp deletion encompassing RBE' in the C-C' region (Figure 2F). It is referred to the 119-bp deleted ITR. As discussed later, AAV plasmids carrying one wildtype and one deleted ITR, but not both deleted ITRs, can still be packaged into virus. Note that Figure 2E and 2F depict single-stranded DNA secondary structure based on the sense strand of the ITR sequence on the AAV transfer plasmid, rather than the actual ITR sequence in the recombinant AAV genome being produced upon virus packaging.

To comprehensively assess the fidelity of ITRs on AAV transfer plasmids, we analyzed 338 AAV transfer plasmids (including 2 self-complementary AAV) sourced from academic and industrial labs worldwide. We first subjected them to RE digestion using either *SmaI* or *AdhI*, two enzymes with recognition sites in both wildtype and 119-bp deleted versions of ITRs as depicted in Figure 2, along with one or two enzymes that cut at sites away from the ITRs. This detects mutations in ITRs that abolish *SmaI* and/or *AdhI* cut sites. The assay revealed that about 9% of the plasmids (29/338) had inconsistent patterns compared to that predicted from the senders' reference ITR sequences (Figure 2G). The 5' and 3' ITRs of the 305 RE-validated AAV plasmids were subjected to Sanger sequencing, which revealed that approximately 30% (92/305) of the 5' ITRs carried mutations relative to their reference sequences (Figure 2H). Interestingly, 3' ITRs seemed more stable, with only around 4% (13/305) of the plasmids showing mutations relative to reference. Additionally, Sanger sequencing failed for 8% of the 5' and 3' ITRs (Figure 2H), presumably due to their high GC content and complex secondary structure that, in the context of some plasmids, are recalcitrant to Sanger sequencing. Of the 274 AAV plasmids with both ITRs successfully sequenced, only 64% (173/274) had both ITR sequences consistent with the senders' reference (Figure 2I). All counted, about 40% of all the surveyed AAV transfer plasmids had at least one ITR deviating from the wildtype sequence. These results revealed the alarming instability of ITRs in AAV plasmids, especially the 5' ITR.

Stability of ITRs in AAV transfer plasmids is affected by flanking sequences

We examined whether sequences immediately flanking the ITRs on the AAV transfer plasmids would impact their stability. Based on reference 5' ITR sequences from the senders, 274 plasmids were classified into four distinct groups distinguished by the 5' ITR sequence itself and the nature of its upstream flanking sequence (Figure 3). Group A consists of 147 plasmids whose sender-provided 5' ITR reference sequences matched the 130-bp wildtype ITR version

shown in Figure 2E, and additionally, the flanking sequence immediately upstream of the 5' ITR contained an 11-bp high-GC (73%) sequence (Figure 3A). Upon Sanger sequencing, we found that around 61% (90/147) of the Group A plasmids contained mutations in their 5' ITRs relative to the reference (Figure 3A). Among the mutations, the most prevalent, which occurred in 87 out of 90 cases, was the deletion of 11 bp in the C-C' region (Figure 3A, Table S4), which effectively converted the 5' ITR from the 130-bp wildtype version shown in Figure 2E into the 119-bp deleted version shown in Figure 2F. Additionally, 5' ITRs on two plasmids exhibited a 22-bp deletion, and on one plasmid, a 4-bp deletion (Figure S1).

Group B consists of 52 plasmids whose sender-provided 5' ITR reference sequence also matched the 130-kb wildtype ITR version shown in Figure 2E, but the flanking sequence immediately upstream of the 5' ITR had GC content ranging from 9% to 64% (Figure 3B, Table S4). Strikingly, none of the plasmid in this group had a different 5' ITR from its reference except for 3 plasmids whose 5' ITR was not fully sequenced.

Group C consists of 73 plasmids whose sender-provided 5' ITR reference sequence matches the 119-bp deleted version as depicted in Figure 2F, and which also have the same 11-bp high-GC (73%) flanking sequence immediately upstream of the 5' ITR as found in Group A (Figure 3C). Among them, only one contained a mutation that is a 15-bp deletion (Figure S2, Table S4). Lastly, Group D consists of 2 plasmids with the identical 119-bp 5' ITR as the Group C plasmids, yet the flanking 11 bp contain 18 or 36% GC (Figure 3D), and none are mutated.

Thus, the coupling of the wildtype version of 5' ITR with high-GC flanking sequence appears to lead to greatly increased mutability, with the 11-bp deletion in the C-C' region being the most prevalent mutation. This hypothesis aligns with previous finding of ITR instability on AAV plasmids when flanked by a 15-bp sequence of 100% GC, which was markedly improved when this flanking sequence was eliminated[9]. It also aligns with our own experience that during cloning, using AAV plasmid backbones in which ITRs are flanked by high-GC sequences tends to produce more clones bearing mutations in the ITRs. Indeed, when we cultured E. coli carrying a plasmid with two validated 130-bp ITRs flanked by the 11-bp high-GC sequence as shown in Figure 3A for ten passages, we found that about half of the plasmid DNA now carried the 119-bp deleted version of 5' ITR as shown in Figure 2F, indicating a remarkably high mutation rate (unpublished data).

The above observations also argue that the 119-bp deleted version of 5' ITR in Figure 2F, even when annotated in the reference sequence as such, was not created intentionally for a purpose by someone, but actually resulted from frequent deletions occurring to the 130-bp wildtype ITR in Figure 2E when flanked by the 11-bp high-GC sequence that caused instability to the ITR. This prevalent mutation probably occurred independently in multiple labs, which then likely passed onto many other labs. It is possible that some researchers were unaware of this mutation having occurred in their AAV plasmids and still assumed the wildtype 5' ITR sequence as the reference, while some other researchers saw the mutated sequence at some point and just considered it to be the correct reference. The same 119-bp deleted 5' ITR were also observed in two plasmids for which the 11-bp upstream flanking sequence contained low GC (18% or 36%). For these, it is possible that the 11-bp deletion occurred spontaneously in their 5' ITRs even in the context of low-GC upstream flanking sequence, or the deletion first occurred on a different backbone containing the high-GC flanking sequence, and the ITR-to-ITR region was later subcloned into the current vectors.

We next examined the integrity of 3' ITRs in AAV transfer plasmids. In Group A, which contained 147 plasmids, 143 sender-provided 3' ITR reference all bore the same 130-bp sequence and the 11-bp high-GC flanking sequence, just like their 5' ITR reference sequence (Figure 3A). We found that 8 plasmids in this group had point mutations in their 3' ITR, while none had mutations

in their 5' ITRs. Based on the senders' reference, the 3' ITR on two of the remaining four vectors was of the 119-bp deleted version, flanked by the high-GC 11-bp sequence. The 3' ITR of the last two plasmids was of the 130-bp version, whose 11-bp flanking sequence had 64% GC. These four vectors had no mutations in their 3' ITR relative to the senders' reference. In Group C with 73 plasmids, the sender-provided 3' ITR reference sequence all had the 130-bp wildtype version, with 72 having the high-GC 11-bp flanking sequence and only one having a low-GC (18%) 11-bp flanking sequence. In four of these plasmids, the 3' ITRs also mutated to the 119-bp deleted version, such that both their ITRs were of the deleted version. No mutations were found in the 3' ITR of Groups B and D plasmids.

There are two important take-home messages from the above data. First, the same 130-bp ITR sequence with the 11-bp high-GC flanking sequence can be exceedingly mutable when it is the 5' ITR, and moderately mutable when it is the 3' ITR (Figure 3). This suggests that there are other factors affecting ITR stability, which we hypothesize to be the distance from the ITR to the plasmid replication origin (Ori). The 5' ITR is usually 200-500 bp from Ori, whereas the 3' ITR is typically over 2 kb away from Ori. Second, both ITRs of a transfer plasmid can be mutated, and when this happens, virus packaging is severely impaired as discussed below.

It has been shown that when packaged into virus, AAV transfer plasmids carrying a mutant ITR on one end and a wildtype ITR on the other end can produce viral genome for which the mutant ITR is repaired, presumably by templating off of the wildtype ITR[14]. This notwithstanding, how different types of mutant ITRs influence packaging efficiency, viral genome integrity, and intended biological functions of the virus is not well understood. Furthermore, once mutations have occurred to one ITR, such as the 11-bp deletion that converts the 130-bp wildtype version to the 119-bp deleted version, additional mutations can still happen to the other ITR at a reasonable frequency. When both ITRs are mutated, the repair mechanism is no longer effective, and AAV packaging will be seriously compromised[11]. Caution is thus advised when using AAV vectors in gene therapy applications where ITR fidelity could impact drug efficacy and safety. We suggest that AAV transfer plasmids whose 5' and 3' ITRs are both the wildtype version, and which do not show a strong tendency to mutate (such as the high mutability observed for the ITR with the high-GC flanking sequence), are preferrable over other designs in gene therapy applications.

DISCUSSION

For many decades, researchers have made their own customized plasmids in the lab to meet their specific research needs. Despite the ubiquity and critical importance of lab-made plasmids in research and medicine, there is as yet no systematic assessment of their quality.

Being a cloning service provider, we had the opportunity to handle a large number of plasmids sourced from academic and industrial labs around the world. We report, for the first time, a large-scale quality assessment of lab-made plasmids, which showed, much to our surprise, a high rate of errors. We found that approximately 15% of plasmids had significant design errors, and about 35% contained sequence errors in functional regions (excluding AAV ITRs) (Figure 1). For AAV transfer plasmids, about 40% had mutations in their ITRs relative to the wildtype form. In total, we estimate that 45-50% of lab-made plasmids have undetected design and/or sequence errors that could potentially compromise the intended applications. Indeed, we suspect that this figure may underestimate the true scale of quality issues in lab-made plasmids because we had asked our clients to check the designs and sequences of their plasmids before submission to us, and also because they were paying for our services utilizing their plasmids.

The high error rate of lab-made plasmids suggests that many labs lack the sophisticated and nuanced expertise needed to properly design vectors and furthermore, there is insufficient quality control of the plasmids being constructed and propagated in labs. Our finding mirrors other reports of major problems with widely used lab reagents that have gone “under-the-radar” for many years simply because researchers did not think to question their quality, such as mycoplasma contamination and misidentified mammalian cell lines[3-5].

We argue that there is a compelling need for community-wide standards and resources to uphold the quality of gene delivery vectors in research and medicine. These may include educational materials on how to design appropriate vectors for various applications, best practices in the construction, propagation, storage, transfer and QC of plasmids and related reagents such as libraries and packaged viruses, and mechanisms that encourage researchers to share their expertise especially tips for improving vector performance and avoiding pitfalls.

METHODS

Sample collection

Our global clients submitted their lab-made plasmids to be modified, used as cloning materials, packaged into recombinant viruses, employed as templates for making RNA by in vitro transcription (IVT), or used in other molecular biology services. These starting materials were required to be submitted as DNA solution (>1 µg dissolved in 0.1-1 X TE) or bacterial stab culture, along with their theoretical vector maps and sequences. For AAV transfer plasmids used for the packaging of recombinant AAV virus, the two ITRs flanking the payload region are in theory identical in sequence and are therefore indistinguishable based on sequence alone. By convention, we refer to the ITR closer to the replication origin on the plasmid backbone as the 5’ ITR (aka left ITR or upstream ITR), and the other ITR as the 3’ ITR (aka right ITR or downstream ITR). Some of our clients labeled their ITRs in the opposite way, which we changed to the above standard convention for consistency.

Sample analyses

Before project initiation, the submitted plasmids were subjected to our standard QC protocols. The designs of the plasmids were manually evaluated by our scientists to ensure their correctness for the intended applications. The structures and sequences of the plasmids were validated by restriction digestion and/or Sanger sequencing. Comparison between Sanger sequencing and other commonly used sequencing methods for validating plasmids such as single-molecule sequencing showed that in cases where discrepant results were produced, Sanger gave the correct sequences. We therefore relied exclusively on Sanger for sequence validation of plasmids. Two well-tested restriction enzymes with theoretical cut sites on the plasmid map were applied together or individually on the plasmid DNA and analyzed with gel electrophoresis. Bands produced were compared to theoretical bands from the submitted plasmid maps and sequences. Regions of the vector critical to biological functions or downstream applications, such as cloning, virus packaging, and IVT RNA production (e.g. cloning sites, coding sequences, promoters, UTRs, PolyA signal sequences, lentivirus LTRs, AAV ITR, etc.), were validated by Sanger sequencing.

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Table 1. Design errors in lab-made plasmids

Design Error	Consequence	Occurrence (out of 2521 vectors)	Fraction (out of 2521 vectors)
Promoter			
Using CMV promoter in AAV vectors intended for ubiquitous expression in vivo	Frequent silencing in host cells in vivo	44	1.75%
Using same promoter to drive two genes in lentiviral vectors	Frequent recombination between promoters leading to deletion of sequence between promoters	39	1.55%
Using CAG promoter in lentiviral vectors	Significantly reduced packaging efficiency and viral titer	26	1.03%
Pol III promoter used to drive protein coding genes	Failure of transcription	4	0.16%
ORF			
Insert length far exceeding limited cargo capacity of vector systems such as lentivirus, AAV, and Sleeping Beauty	Decreased efficiency and/or decreased packaging and viral titer	23	0.91%
Vectors containing toxic genes to E. coli host	Decreased growth or instability and accumulation of mutations	21	0.83%
Placing expression cassette driven by ubiquitous promoter in antisense direction in lentiviral vectors	Transcription of transgene clashes with transcription of viral genome, reducing viral titer	18	0.71%
Viral vectors containing toxic genes to virus packaging cells	Decreased packaging efficiency and resulting viral titer	12	0.48%
Placing recombinase gene and recombinase recogition sites in the same vector (e.g. Cre-lox, Flp-FRT and Dre-rox)	Unintended recombination at recognition sites in E. coli and/or packaging cells	5	0.20%
Stop codons / polyA signals			
Extra stop codon in ORF upstream of 2A	Translation terminated before reaching the second ORF	31	1.23%
No stop codon in ORF upstream of IRES	Extraneous amino acids added to the C terminus of upstream ORF	14	0.56%
PolyA signal placed internal of lentiviral vectors in the sense direction	Premature termination of transcription of the lentiviral genome during packaging, decreasing viral titer	7	0.28%
No polyA signal after ORF (excluding lentiviral vectors)	Compromised gene function	5	0.20%
RNA hairpin			
miR30-based shRNA not including small mismatch between sense and antisense sequences to mimic the original miR30 structure	Inefficient knockdown	11	0.44%
shRNA with flipped sense and antisense sequences in the hairpin	Compromised knockdown efficiency	7	0.28%
Regulatory element			
Kozak not included or not placed immediately upstream of ORF	Decreased translation efficiency	17	0.67%
CRISPR donor vector not mutating PAM sequence	Donor vector becomes target of CRISPR cleavage	9	0.36%
CRISPR gRNA vector wrongly including PAM sequence	gRNA becomes target of CRISPR cleavage	7	0.28%
Other			
Highly unstable sequences (e.g. high GC, large hairpin, and long repeats of trimers, dimers and monomers) placed in vectors with high-copy plasmid orgin	Instability and accumulation of mutations	27	1.07%
Other miscellaneous errors		57	2.26%
	Total	**384**	**15.23%**

Figure 1

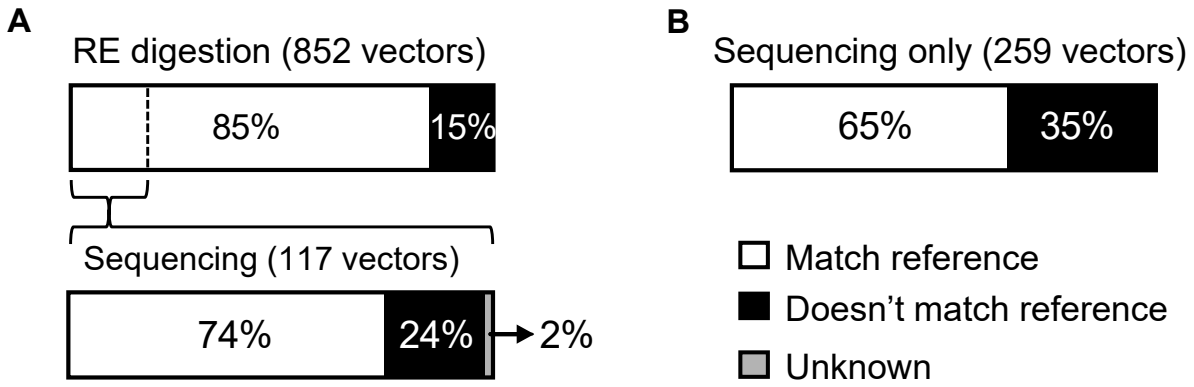

Figure 1. High rate of sequence errors in lab-made plasmids from global researchers. (**A**) Error rate of plasmids was assessed by restriction enzyme (RE) digestion, and a subset of RE-validated plasmids were further assessed by Sanger sequencing of their functional regions. (**B**) Error rate was directly assessed by Sanger sequencing of functional regions without RE digestion.

Figure 2

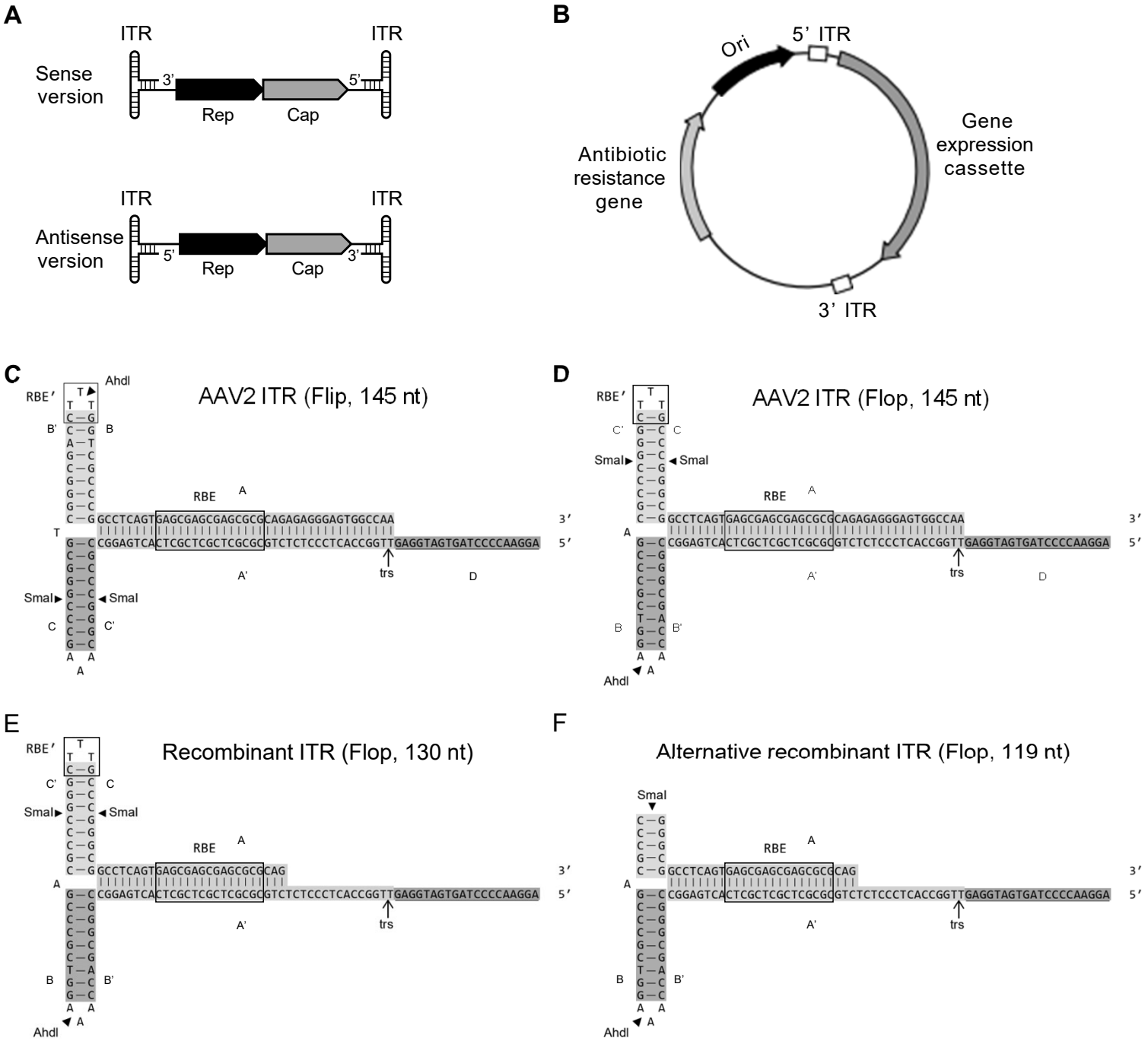
A
ITR
ITR
Sense version
3'
5'
Rep
Cap
ITR
ITR
Antisense version
5'
3'
Rep
Cap
B
Ori
5' ITR
Gene expression cassette
Antibiotic resistance gene
3' ITR
C
AAV2 ITR (Flip, 145 nt)
RBE'
Ahdl
RBE
A
A'
trs
D
Smal
Smal
D
AAV2 ITR (Flop, 145 nt)
RBE'
Smal
Smal
RBE
A
A'
trs
D
Ahdl
E
Recombinant ITR (Flop, 130 nt)
RBE'
Smal
Smal
RBE
A
A'
trs
Ahdl
F
Alternative recombinant ITR (Flop, 119 nt)
Smal
RBE
A
A'
trs
Ahdl

Figure 2 (continued)

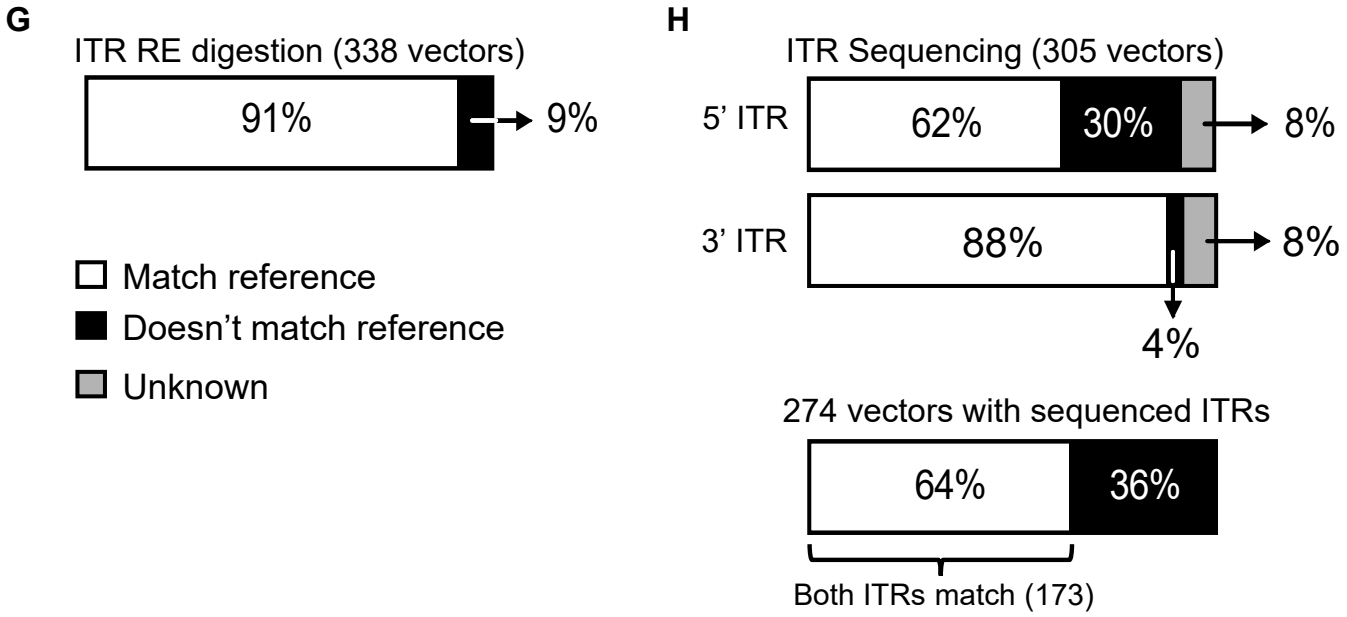

Figure 2. High mutability of ITRs in AAV transfer plasmids. The AAV2 viral genome is a ~4.7 kb, single-stranded DNA containing Rep and Cap genes flanked by two ITRs on its ends that reverse complement each other (**A**).The typical recombinant AAV transfer plasmid contains two ITR regions flanking the expression cassette (**B**). The secondary structure of the 145-nt AAV2 viral genome ITR is shown in either flip (**C**) or flop (**D**) configuration. It contains three self-annealing regions, A-A', B-B', and C-C', and a single-stranded extension, D, before reaching the internal sequence of the viral genome. The A-A' stem contains the Rep-binding element (RBE) and the terminal resolution site (trs). The C-C' arm contains a secondary Rep-binding element (RBE'). The ITRs of AAV transfer plasmids typically have three versions. One is 145 bp long that corresponds to the flop version of ITR in the AAV2 viral genome (**D**). The second is 130 bp long and corresponds to the AAV2 viral genome ITR minus the terminal 15 nt (**E**). The third has an additional 11-bp deletion and is 119 bp long (**F**). The integrity of B-B' or C-C' arms on recombinant AAV transfer plasmids can be assayed by restriction enzyme (RE) digestion using *AhdI* or *SmaI*, respectively. (**G**) ITR integrity of 338 AAV transfer plasmids as assayed by RE digestion. (**H**) Integrity of either 5' or 3' ITR in the 304 AAV plasmids with correct RE digestion, as further assayed by Sanger sequencing. (**I**) Combined integrity of 5' and 3' ITRs in the 270 plasmids for which both ITRs were sequenced.

Figure 3

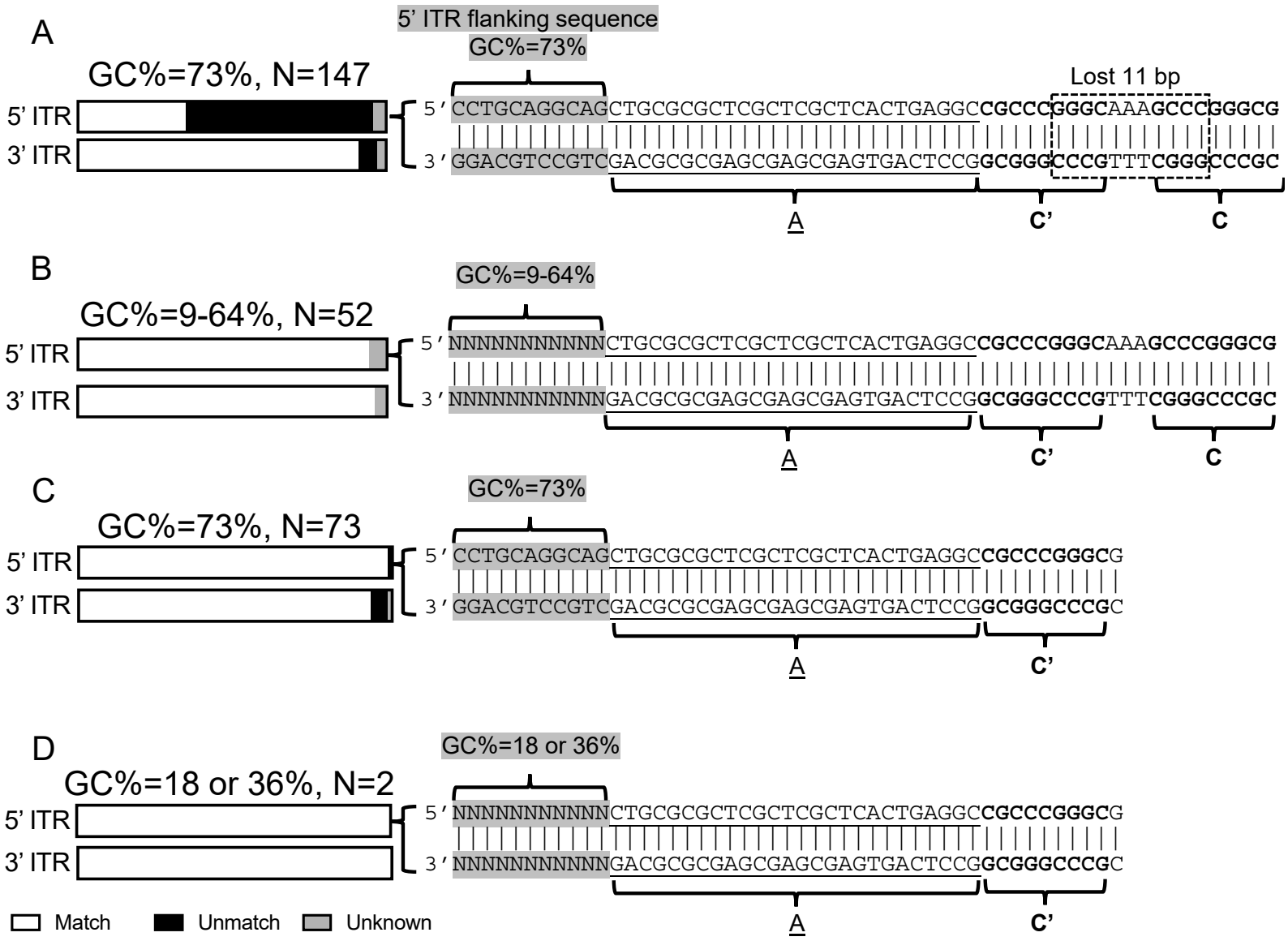

Figure 3. Correlation between ITR stability and GC content in the 11-bp flanking sequence of three groups of 5' ITR. (**A**) 5' ITR in 147 plasmids have a high GC content (73%) 11-bp flanking sequence, and the sequence of 90 5' ITR (~61%) were unmatched with the user-provided reference. 87 of the unmatched 5' ITR lost the 11 bp in the C arm (boxed with dash line) compared to their reference. (**B**) 52 plasmids have the exact same 5' ITR as (**A**) but 9-64% GC content in the 11-bp flanking sequence. No mutation was detected in the 5' ITR of these plasmids. (**C**) 73 plasmids had the exact same high-GC 11-bp flanking sequence but the alternative 5' ITR sequence missing the 11 bp in the C arm. The 5' ITR sequence of one plasmid unmatched its reference. (D) 2 plasmids had the alternative 119-bp 5' ITR but different flanking 11 bp sequence of lower GC content.