nature structural & molecular biology

Perspective

https://doi.org/10.1038/s41594-025-01492-x

Guidelines for minimal reporting requirements, design and interpretation of experiments involving the use of eukaryotic dual gene expression reporters (MINDR)

Received: 7 February 2024

Accepted: 20 January 2025

Published online: 03 March 2025

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Dual reporters encoding two distinct proteins within the same mRNA have had a crucial role in identifying and characterizing unconventional mechanisms of eukaryotic translation. These mechanisms include initiation via internal ribosomal entry sites (IRESs), ribosomal frameshifting, stop codon readthrough and reinitiation. This design enables the expression of one reporter to be influenced by the specific mechanism under investigation, while the other reporter serves as an internal control. However, challenges arise when intervening test sequences are placed between these two reporters. Such sequences can inadvertently impact the expression or function of either reporter, independent of translation-related changes, potentially biasing the results. These effects may occur due to cryptic regulatory elements inducing or affecting transcription initiation, splicing, polyadenylation and antisense transcription as well as unpredictable effects of the translated test sequences on the stability and activity of the reporters. Unfortunately, these unintended effects may lead to misinterpretation of data and the publication of incorrect conclusions in the scientific literature. To address this issue and to assist the scientific community in accurately interpreting dual-reporter experiments, we have developed comprehensive guidelines. These guidelines cover experimental design, interpretation and the minimal requirements for reporting results. They are designed to aid researchers conducting these experiments as well as reviewers, editors and other investigators who seek to evaluate published data.

Gene fusions expressing reporters have been used to characterize mRNA translation mechanisms since the 1990s[1–5]. The popularity of this approach increased with the invention of the dual luciferase reporter in 1998 (ref. 6). Since then, dual reporters have been used to study ribosomal frameshifting, stop codon readthrough, reinitiation and internal initiation[6–12]. Dual-reporter systems have proven instrumental, in combination with site-directed or random mutagenesis, in characterizing mRNA features involved in specific translation mechanisms[11,13–23] and for the identification of underlying cellular factors impacting these mechanisms[24–32]. Dual reporters have also found application in the identification and study of drugs targeting specific mechanisms of translation[25,33–39] as well as the mechanisms of disease-associated polymorphisms[40]. Increasingly, dual reporters are being used to discover novel translational mechanisms and processes[41,42].

The principle is based on encoding two distinguishable reporters within the same mRNA transcript. This design ensures that the

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https://doi.org/10.1038/s41594-025-01492-x

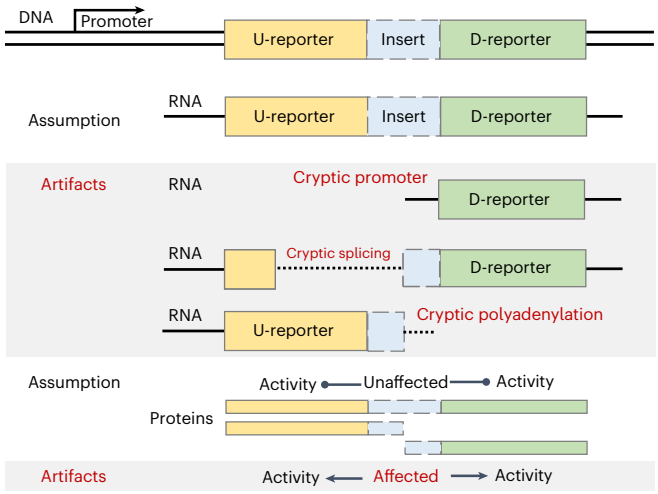


Fig. 1 | Principles of a dual-reporter strategy and the most common artifacts (shaded background) arising from unexamined assumptions. It is expected that a single RNA species is transcribed from the DNA sequence shown at the top of the illustration. Even if a single RNA species is transcribed, it is assumed that the fusion of the insert sequences to either reporter does not alter reporter protein activity or stability. However, several shorter possible RNA species are shown that could lead to artifactual reporter expression. D-reporter, downstream reporter; U-reporter, upstream reporter.

expression of both reporters is coupled because they are transcribed together. One reporter reflects the activity of the mechanism under evaluation. The second reporter serves as an internal control to normalize for any variations in mRNA levels or experimental conditions (Fig. 1). As reporter proteins are synthesized from the same mRNA, it is assumed that differences in the expression of the reporter used to characterize the mechanism under study are independent of confounding variables, such as transfection efficiency or variations in RNA levels and/or stability. The assay can be performed in a single tube or a well with the ratio of upstream-to-downstream reporter activity as a readout. However, differences in reporter expression can occur for several reasons other than differential translation, potentially leading to misinterpretation of reporter readout (Fig. 1). Furthermore, because the readout of dual-reporter assays is usually the ratio of downstream-to-upstream reporter activity, changes in the upstream internal control reporter without changes to the downstream reporter could lead to misinterpretations unless absolute reporter values are carefully considered.

There are various possible sources of dual-reporter artifacts. First, DNA-encoded dual reporters may contain cryptic promoters, cryptic splice sites or cryptic polyadenylation signals that could generate unexpected mRNA transcripts encoding only one of the two reporters[11,43–54]. Plasmid DNA can also produce unexpected antisense transcripts that may affect reporter expression[55]. Second, the protein extensions encoded by the test sequence may alter the stability or activity of one or both reporters, if it is synthesized as a part of the same polypeptide chain[12,56]. Finally, in certain applications, the downstream reporter is placed under a known (control) or a putative (test) IRES. However, IRES activity may be influenced by surrounding sequences[20,21,57,58] and it has been shown that the presence of an IRES may influence mRNA stability[59] and translation[60,61] of the upstream reporters in a sequence-dependent manner.

Although various artifacts generated by experiments involving dual reporters have been discussed extensively[11,12,46–50,52,57,62–70], the misinterpretation of these assays continues to result in inaccurate conclusions[53,54,56].

Therefore, we developed guidelines for the design, interpretation and reporting of experiments involving single-mRNA-encoded dual reporters. In the first section of this Perspective, we discuss the principles and recommendations related to the design and interpretation of dual-reporter experiments. We provide guidance on the selection of appropriate vectors to reduce the risks of false positive findings and the design of critical controls and additional experiments that may help to identify and avoid erroneous conclusions for a wide range of mechanisms. In the second section, we will discuss minimal information on dual expression reporters (MINDR), which outlines proposed reporting requirements that establish the essential data that should accompany any publication describing dual-reporter experiments. MINDR is designed to facilitate reproducibility and provide editors, reviewers and readers with the information necessary for evaluating the reliability of the dual-reporter strategies that have been implemented and the assessment of the likelihood of false positive findings.

Principles and recommendations

In this section, we consider reporter design, possible controls and data interpretation. These are intended to equip researchers with the means to investigate and avoid potential artifacts. We first describe general aspects regarding most dual-reporter applications, followed by considerations relevant to specific types of assays or phenomena arising from experiments, which are also summarized in Table 1.

General sources of artifacts

Unexpected mRNA species. *Assumptions and potential problems.* It is often assumed that all measured reporter activity is derived from translation of a single mRNA species generated in strict accordance with the researcher's design. However, unexpected mRNA species may be produced through either cryptic splicing, cryptic transcription or internal polyadenylation (Fig. 1). These aberrant monocistronic transcripts could be translated much more efficiently than their bicistronic counterparts and, even if they represent only a tiny fraction, could substantially distort the overall interpretation of the results[11,44–46,49–54].

Recommendations. The most straightforward way to control for these possibilities is to introduce in vitro transcribed RNA encoding the reporter proteins instead of using DNA-based constructs. Any discrepancies between DNA-encoded and RNA-encoded reporter protein ratios may indicate the existence of unexpected cryptic transcripts, although there may be other factors explaining these differences as discussed below. There may also be situations when RNA transfections are impractical as described in RNA versus DNA reporters.

For DNA transfections using reporters that are expected to express fusion proteins, for example, by readthrough or frameshifting, western blotting is an effective and sensitive approach to ensure that downstream reporter protein expression is not derived from aberrant mRNA species producing shorter variants of fusion proteins.

Where products of dual reporters are not expected to be fused, for example, in StopGo vectors and reinitiation or IRES studies, it may be desirable to identify possible aberrant mRNA species directly. Because even a minor mRNA species could produce a major signal if translated very efficiently, highly sensitive techniques for detecting low-abundance mRNA species are required. To this end, emerging long-read sequencing technologies (such as nanopore or PacBio) or quantitative PCR with reverse transcription (RT–qPCR) may be sufficiently sensitive approaches to detect low-abundance RNA species, whereas northern blotting may not be sensitive enough. RT–qPCR can be used to assess the levels of different RNA segments[46] with specific primers as shown (Fig. 2). Careful design and validation of RT–qPCR amplicons with in vitro transcript standards and melting curves are necessary to determine whether the amounts of each amplicon are equal[71]. As the same PCR amplicons can be derived from alternative transcripts and because RT–qPCR optimization is not always straightforward, it is advisable that this method is not the sole approach used

https://doi.org/10.1038/s41594-025-01492-x

Table 1 | Recommended controls and orthogonal evidence

Phenomenon	Negative control	Positive control	Orthogonal evidence
Frameshifting	Mutate putative frameshift site; introduce stop codon downstream of frameshift site in frame with downstream reporter.	Introduce an indel to place reporters in same reading frame and make synonymous changes to the slip site.	Immunoblotting if reporters are fused; ribosomal profiling: when frameshifting produces longer proteoform, ribosome footprint density is expected downstream of zero frame stop codon with triplet periodicity phase matching new frame. When frameshifting is efficient and is predicted to produce a shorter proteoform, drop of ribosome footprint density is expected downstream of stop codon in the new frame.
Stop codon readthrough	Introduce double stop codon (for example, two UAA codons instead of a readthrough stop codon candidate).	Stop-to-sense codon substitution.	Immunoblotting if reporters are fused. Ribosome profiling: ribosome footprint density is expected downstream of the stop signal with triplet periodicity matching the zero frame.
Reinitiation	Overlap between upstream and downstream reporter ORFs; introduce a stop codon or indel in the intercistronic region closer to the second cistron.	For reinitiation after short uORFs, delete AUG of an upstream uORF; for long uORFs, reporters could be fused in frame and separated with StopGo.	Immunoblotting to show that reporters are not synthesized as a single fusion protein. Use several different reporters as a second cistron.
Internal initiation	In DNA construct, delete the promoter directing the bicistronic transcript. Compare with bicistronic constructs harboring long nonspecific intergenic sequences.	Compare with known effective viral IRES, for example, encephalomyocarditis virus, and with the corresponding monocistronic constructs.	mRNA transfection instead of DNA reporters; compare bicistronic construct with the corresponding monocistronic ones (m^7G capped, A capped); insert 5′-terminal stem-loop and/or long overlapping uORF; siRNA targeting upstream reporter; polysome analysis; map the transcription start sites of the downstream reporter using 5′ RACE; using appropriate cell-free systems.

Abbreviation: 5′ RACE, 5′ rapid amplification of cDNA ends.

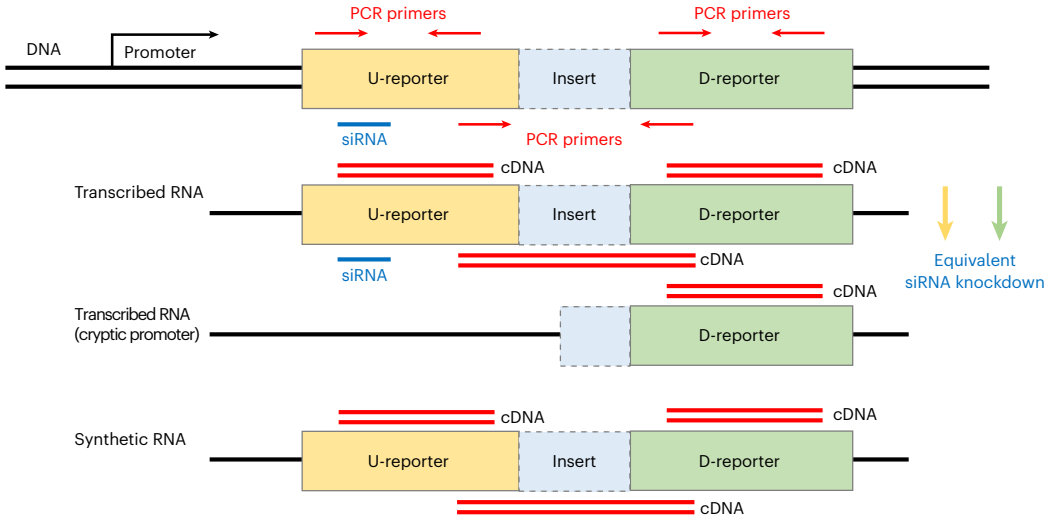


Fig. 2 | Assessment of dual-reporter transcriptional integrity. Red arrows show the design of forward and reverse primers for the dual-reporter construct, and double red lines depict the resulting cDNA products. Using a synthetic RNA construct (bottom), it is possible to estimate the ratio between cDNA products for the transcript containing all three regions. Horizontal blue lines represent siRNAs for knockdown experiments expected to equivalently downregulate expression of both reporters if they are produced from the full-length RNA template as indicated with vertical yellow and green arrows.

to analyze the occurrence of transcript isoforms[68]. An in vitro transcribed full-length control RNA can be used to assess the signal expected from a single RNA species containing both reporters (Fig. 2).

Furthermore, an elegant approach using RNA interference can be used to regulate the authenticity of bicistronic RNA[46]. A small interfering RNA (siRNA) probe designed to knock down expression of the upstream reporter directs specific degradation of the bicistronic reporter mRNA, thereby decreasing the activity of both the upstream and downstream reporters to a similar extent, unless they are expressed from different mRNA species (Fig. 2). Transcript isoforms may also be explored by methods like cap analysis of gene expression (CAGE)[72] or with direct RNA sequencing using nanopore technologies, bearing in mind that low-abundance alternative products can produce most of the reporter activity.

Potential existence of aberrant transcripts may also be explored with study-specific controls, the design of which is specific to the studied phenomena.

Altered activity or stability of fused reporter proteins. *Assumptions and potential problems.* When testing relative activities from fused reporter proteins, for example, for readthrough or frameshifting, it is often assumed that the reporter protein activities accurately reflect reporter mRNA translation. Frameshifting and stop codon readthrough efficiencies are generally estimated by determining the ratio of upstream-to-downstream reporter protein of a test construct relative to the reporter protein ratio from a positive control (100%), where both reporters are in the same, uninterrupted reading frame. However, the part of the protein fusion encoded by the test sequence

https://doi.org/10.1038/s41594-025-01492-x

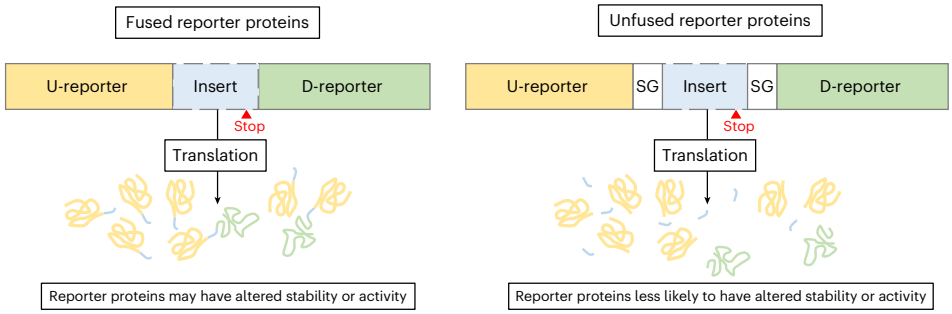


Fig. 3 | Fused versus unfused dual reporters. Both systems have their merits and drawbacks. The advantage of fused reporters is the ability to estimate recoding efficiencies by western blotting or in vitro translation reactions while controlling for unintended downstream reporter translation by internal initiation or cryptic splicing. One potential downside of fused reporters is erroneous estimations of recoding efficiencies based solely on reporter protein activities because of possible changes in downstream reporter activity resulting from its fusion to the test and upstream reporter proteins. This can be mitigated with unfused reporter systems in which the test peptide is released from the reporter proteins by flanking StopGo (SG) motifs. While western blotting or in vitro translations can still be used to estimate recoding efficiencies with unfused reporter systems, due to the similar-sized downstream reporter protein produced, spurious downstream reporter protein expression by internal initiation or cryptic splicing may not be as obvious.

may influence the folding, stability or activity of one or both reporter proteins. This can be especially problematic if fusions to the upstream reporter only influence the in-frame control reporter protein and not the test reporter or vice versa (Fig. 1)[12,56].

Recommendations. Place StopGo/2A sequences on both sides of the test sequence[12] (Fig. 3). StopGo is an atypical translation mechanism in which the elongating ribosome fails to form a peptide bond and then efficiently continues translation; the outcome of this phenomenon is the production of two peptide chains from the same open reading frame (ORF) separated by the StopGo motif[73].

A peptide motif enabling StopGo was initially discovered during translation of the 2A region of foot-and-mouth disease virus[74], and, since then, a number of 2A peptides with varying efficiencies have been described. While in theory StopGo use should produce reporter proteins with identical amino acid sequences irrespective of the inserted test sequence, this may not always be the case. StopGo is not 100% efficient (generally 80–90%[75]); therefore, a certain amount of fusion between either of the reporters and the test sequence-encoded fragment will still be produced. Nonetheless, for example, a comparison of absolute *Renilla* luciferase activities (when used as an upstream reporter) from a series of fused and unfused test constructs indicated much more variability with fused reporters than with the StopGo-containing plasmid[12]. Additionally, the potential for ribosome drop-off during StopGo that may influence the ratio between two reporters can be easily tested with an appropriate in-frame control construct. When using a new StopGo-containing plasmid, it is important to monitor the efficiency of StopGo by western blotting in the intended biological system. Although variability in StopGo activity has been reported[76], it can be mitigated by using longer StopGo motifs (>30 amino acids), which are less sensitive to the effects of proximal sequences, including test inserts, while having higher activities and negligible ribosome drop-off[75]. It should be noted that, although StopGo is active in all eukaryotes tested so far, different 2A peptide variants have different activities in various organisms, and none have been found to function in bacteria[77]. Furthermore, the kinetics of StopGo are still poorly understood and it is likely to cause some ribosome pausing, which may interfere with some of the studied phenomena.

Alterations in reporter ratios due to artifactual changes in reporters' absolute readouts. *Assumptions and potential problems*. It seems reasonable to assume that, when transfecting the same reporter construct into two different cell lines or under different conditions, the ratiometric readout should be similar. However, this is not always the case. Not surprisingly, absolute reporter values may be quite different between cell lines and conditions, sometimes spanning orders of magnitude. There may be several reasons for this, including differences in reporter delivery efficiencies between cell lines. Reporter RNAs may trigger the innate immune response, resulting in global suppression of translation, for example, through activation of protein kinase R (PKR), cyclic GMP–AMP synthase (cGAS), Toll-like receptors (TLRs), NOD-like receptors (NLRs) or RIG-I-like receptors[78,79]. Reporter expression in particular cell lines can be compromised at either the transcriptional level (for example, weak promoter activity in DNA reporters) or the translational level (for example, low global translation rates). When dual reporters are used to compare the efficiency of the studied mechanism across cell lines or under specific conditions (including stress versus control, overexpression or depletion of specific factors, treatment with small-molecule inhibitors and nonsense-mediated decay (NMD) activation), it is important to understand that differences in global translation may affect the ratio of the measured reporter activities without affecting their real relative activities. This is because any measured activity is a combination of genuine reporter protein activity with background levels due to biological noise and technical limitations. We can represent the ratio of measured activities as $(X+n)/(Y+n)$, where X, Y are bona fide activities of the two reporter proteins and n is the constant background signal. Therefore, if X and Y are changed proportionally (for example, halved by general translation inhibition) and the X/Y ratio stays the same, the measured $(X+n)/(Y+n)$ ratio will be altered.

Perhaps more problematically, there may be cell-specific differential reporter activity due to the presence of a cell-specific repressor or activator that acts on one of the reporters. Another factor to consider is reporter overexpression driven by a strong promoter that could further exacerbate cell-specific effects by, for example, titrating essential translation components.

Recommendations. Here it is important to report absolute readouts of reporter and background activities to reveal potential misinterpretations of observed changes in relative activities of the reporters, for example, as previously described[53].

Assay-specific principles and recommendations

RNA versus DNA reporters. The use of mRNA transfection is a powerful strategy to avoid the generation of unexpected RNA species by cryptic transcription, polyadenylation and/or splicing events. However, its advantages are not limited to this. For several applications, RNA transfection may be preferred irrespective of transcriptional artifacts. Analysis of the immediate stress response is particularly difficult when using plasmid DNA reporters. Applying stress stimuli immediately after DNA transfection is ineffective, as substantial time

is required for sufficient mRNA accumulation. By contrast, for RNA transfection, the stress stimuli can be applied immediately or shortly before or after transfection (1–2 h); here, newly synthesized protein products are responsible for most of the reporter activity. It is also difficult to synchronize the expression of DNA reporters in the entire cell population without synchronizing their cell cycle, as the plasmid only enters the nucleus during mitosis. Furthermore, transfection of nondividing cells, for example, matured neurons or cardiac myocytes, with plasmid DNA is highly inefficient.

Nonetheless, RNA transfection is not a panacea and should be used appropriately. First, activity values are generally much lower from mRNA transfections than from DNA transfections. For this reason, RNA transfections should use reporters with a high signal-to-background ratio such as luciferase reporters. Second, RNA is relatively unstable; so it is advantageous to analyze activity shortly after transfection, ideally within 1–4 h after transfection. At later time points, for example, 8–10 h after transfection, accumulated reporter products start to exceed newly synthesized ones, even more so than in the case of DNA transfections. It is also important to use a transfection protocol that minimally stresses the cells and to avoid stress stimuli like cell replating, electroporation or cooling the plate or dish immediately before or during transfection[80]. Finally, as delivered artificial mRNA has not passed through the nucleus, it may lack specific and potentially critical features including epitranscriptomic marks or associated mRNA-binding proteins of nuclear origin. Furthermore, in many cell types, including primary cells, mRNA transfection may trigger innate immune responses; so additional efforts are needed to reduce its activation, like the use of transcripts with a cap1 (having a 2′-*O*-methylated 5′-terminal position) and modified nucleotides (for example, $m^1\Psi$ (N^1-methylpseudouridine) in place of U)[78,79]. It is also important to note that lipocationic transfection impedes the analysis of reporter mRNA stability, as only a tiny fraction of delivered mRNA is released from endosomes into the cytosol[81]. Accordingly, total RNA extraction from the transfected cells yields mRNA that is not predominantly derived from the cytosolic fraction but from endosomal and endolysosomal compartments[81,82]. Finally, delivered RNA may still be processed by cytoplasmic RNA-cleaving enzymes, such as IRE1 (refs. 83,84) or RNase L[85], and other processing events[86]. Therefore, one cannot exclude the possibility that, even in the case of mRNA transfection, aberrant RNA species may also be present. A more detailed comparison of DNA and RNA transfections can be found in a dedicated review[87].

The use of dual reporters for massively parallel assays. In recent years, dual-reporter vectors have become popular in massively parallel reporter assays (MPRA) that simultaneously evaluate thousands of test sequences. This is a powerful approach that allows screening of a diverse pool of sequences for specific regulatory properties, for example, driving internal initiation[41,42], ribosomal frameshifting[34] and other translation mechanisms. It can also be applied to screen a pool of all possible variants of a particular sequence to comprehensively characterize *cis*-acting regulatory elements.

While many of the general principles that should guide the use of dual reporters outlined above also apply here, the high-throughput nature entails several specific considerations associated with measuring reporter protein activity in a pooled manner. Certain guidelines such as reporting absolute measured expression levels of the reporter genes are typically harder to achieve than in the case of single-reporter measurements. On the other hand, dual-reporter MPRAs also allow for the inclusion of a much larger number of controls that can be measured in the same experiment.

Particularly suitable for MPRAs are dual fluorescent reporter constructs, as they allow fluorescence-activated cell sorting based on relative reporter expression levels. This is then followed by DNA sequencing-based identification and quantification of the underlying sequences.

An important consideration for MPRA is to ensure equal vector copy number and comparable expression levels among the cells. The use of systems for genome integration such as landing pads, instead of random integration with lentiviruses, allows the insertion of a single copy of the test vector at the same genetic locus for all cells[88]. However, this may not be strictly necessary. A recent detailed examination[89] revealed a high degree of correlation between the expression of two cistrons across a polyclonal cell population, regardless of the integration site or number of integrated copies of the bicistronic construct.

A substantial challenge in screening large pools of diverse sequences is discerning artifacts amid the positive hits. Reliance on validating only a subset of hits can lead to misinterpretation, as each individual sequence may possess unique properties, and even a single nucleotide change can alter cryptic promoter activity. Given that MPRA-based screens typically yield numerous hits, it is impractical to validate each one individually, making it difficult to estimate the rate of false positives[41,42]. Therefore, general conclusions should not be drawn from limited validated cases. Instead, conducting a meta-analysis of positive hit sequences may help to identify shared features and specific types of artifact. Rare artifacts are unlikely to substantially impact conclusions drawn from MPRAs aimed at characterizing general sequence properties influencing a specific mechanism because of averaging. However, claims related to specific individual sequences in MRPA assays should be limited to those that have been appropriately validated to minimize false positive discovery.

Cell-free translation systems and other in vitro assays. Another potential source of false positives from dual-reporter assays is inappropriate use of in vitro translation systems. For example, the commercially available and widely used nuclease-treated rabbit reticulocyte lysate (ntRRL), which is prepared from specialized cells with a limited range of RNA-binding proteins, has been repeatedly shown to fail in accurately reproducing conditions found in normal cells[62,69]. Exogenously added mRNAs translated in ntRRL exhibit a relatively weak reliance on the 5′ cap[51,90]; although optimizing the buffer conditions can substantially increase cap dependency and start site recognition[91]. Moreover, as some eukaryotic initiation factor 4G (eIF4G) molecules are sequestered by the capped 5′-terminal mRNA fragments remaining in the ntRRL after limited hydrolysis of endogenous reticulocyte transcripts, the addition of cap-dependent initiation inhibitors (such as m^7GTP, 4E-BP or proteases that cleave eIF4G) may release this factor and artificially stimulate translation of uncapped mRNAs. This system also does not recapitulate cap–poly(A) synergy[92]. In sum, this is often misinterpreted as an indication of cap-independent translation of a particular studied mRNA. In addition, ntRRL is prone to aberrant internal initiation at AUG codons located within extended unstructured regions[93], causing artificial expression of the second cistron in bicistronic reporters even in the absence of bona fide IRESs. Finally, translation in rabbit reticulocyte lysate is highly sensitive to even moderately stable RNA secondary structures in the 5′ leader, which is not the case in living cells[90].

It is likely that similar artifacts are present in cell-free systems derived from budding yeast and wheat germ, at least under some conditions[67,94]. By contrast, such effects are not typically observed in cytosolic extracts of cultured mammalian cells[51,90]. However, in any in vitro system, results can greatly depend on the specific preparation conditions and component concentrations. For example, varying polyamine concentrations in the yeast extract can modulate stop codon readthrough efficiency as much as fourfold[95]. Therefore, caution is necessary when comparing findings from a specific cell-free system with cultured cells or in vivo observations.

Less commonly, bicistronic constructs are used in in vitro systems reconstituted from purified components[96,97]. Although such analysis is very informative, it also should be done with caution. Similar to rabbit reticulocyte lysate, in these systems, which are usually devoid

of mRNA-binding proteins, the ribosomes are able to bind to internal AUG codons located within long unstructured regions[98,99]. This risks confusion of an authentic mechanism with an artificial one that should be excluded by validation in complete in vitro or in vivo systems.

Another potential source of artifacts specific to in vitro assays is related to partial hydrolysis of an in vitro synthesized reporter mRNA that can produce truncated versions of bicistronic constructs. Thus, the integrity of the full-length reporter mRNA in both free and ribosome-bound fractions should be extensively analyzed after the assay is complete, for example, by using RT–qPCR with primers targeting either each cistron individually or both cistrons together.

Phenomenon-specific principles and recommendations

Specific considerations in the assessment of ribosomal frameshifting and stop codon readthrough. The assessment of ribosomal frameshifting and stop codon readthrough using dual reporters involves a calculation of the relative reporter activities (downstream-to-upstream ratio). The relative reporter activity of the test construct is then compared to that of a control construct. It is not advisable to calculate reporter activities relative to a negative control rather than a positive control, as this can result in the unintended consequence of a negligible number having an outsized effect. An appropriate positive control has both reporters encoded within the same ORF (in-frame control). Often, a single in-frame control is compared to several test sequences. We caution against this practice when the sequence of the test constructs is considerably different from the sequence of a single in-frame control, as some of the test sequences may contain cryptic splice sites or promoters. The ideal in-frame control should have no amino acid differences from the expected frameshift or readthrough product and minimal nucleotide differences.

For frameshifting, an in-frame control can be obtained with either insertion or deletion of a single nucleotide in the putative frameshifting site depending on the expected direction of frameshifting (−1 or +1). For stop codon readthrough, the stop codon should be replaced with a sense codon, ideally one encoding the same amino acid that is expected to be inserted in place of the stop codon. However, the identity may not be known in advance and there may be one of several possible amino acids inserted[100,101]. Reporter ratios in such positive control constructs are considered to correspond to 100% efficient frameshifting or stop codon readthrough. For studying frameshifting, it is advisable to introduce synonymous changes to disrupt the putative slippery sequence within the in-frame control to create a more accurate readout of 100% frameshifting. If the frameshifting site is not disrupted in an in-frame control and frameshifting occurs (say at 10% efficiency), only 90% of the ribosomes would synthesize the downstream reporter. Thus, ideally, each tested sequence should have its own positive in-frame control. Including a +1 or −1 frame termination codon (depending on the reading frame of the downstream reporter) 5′ of a putative frameshifting site is also worth considering to ensure that only frameshifting within the test sequence is reported.

When using fused reporters, it is desirable to validate frameshifting through orthogonal methods, such as western blotting, when reporting novel instances. Most reporter proteins can be detected by commercially available antibodies that can detect frameshifting or stop codon readthrough efficiencies as low as 1%. As mentioned above, when using fused reporters, western blotting can also control for cryptic splicing and cryptic promoter activity.

Another important way to validate ribosomal frameshifting and stop codon readthrough is the use of a negative control. Ribosomal frameshifting normally occurs at specific frameshifting sites accompanied by stimulatory elements such as specific mRNA structures. While single point mutations in stimulatory signals rarely abolish frameshifting completely, disruptions of the frameshifting site are expected to eliminate frameshifting as transfer RNA repairing in the new frame is precluded. A useful negative control for stop codon readthrough is the insertion of tandem in-frame UAA stop codons that in most organisms represent the most efficient terminators.

Although not always possible, it is desirable to avoid AUG codons within the test sequence that are in the same reading frame as the downstream reporter ORF, as they may serve as initiation codons on cryptic transcripts missing the upstream reporter, entirely or partly. However, deliberate insertion of an AUG codon in frame with the downstream reporter may be used as a control for the existence of cryptic transcripts. In the absence of such transcripts, introduction of an AUG codon in a good Kozak context should not substantially alter the activity of the downstream reporter unless there is reinitiation. Although reinitiation is extremely rare after translation of long ORFs, there are certain signals that could enable reinitiation even after translation of long ORFs[102–105]. Another consideration is potential initiation at near-cognate starts, which is generally inefficient but is known to be highly productive and even comparable to that of AUG in certain contexts; therefore, it is advisable to examine sequences for the presence of such near-cognate start codons in a good Kozak context. Additional evidence may be derived from orthogonal methodological approaches, such as ribosome profiling by accessing publicly available data in dedicated browsers[106,107]. While a detailed discussion of this technique is beyond the scope of this Perspective, we recommend that interested readers consult specialized reviews[108,109].

Specific considerations in the assessment of reinitiation. Reinitiation is a process in which a ribosome initiates translation downstream of the stop codon at which it terminates (reviewed in refs. 110,111). Usually, this process is inefficient in eukaryotes, unless the translated upstream ORF (uORF) is short or mRNA-specific mechanisms are used[102–105,110,112]. However, reinitiation can be greatly facilitated under some physiological stress conditions or when ribosome-recycling factors are artificially depleted[29,113].

The rate of translation reinitiation after long ORFs can be assessed with the dual-reporter assay; other methods are more suitable to examine reinitiation after short uORFs (refs. 110,111 and references therein). However, due to the inefficiency of this process under normal conditions, absolute values of reporter activity and appropriate background correction should be thoroughly considered. As in other cases, cryptic promoters in intercistronic spacers must be excluded and only appropriate cell-free systems should be used for in vitro studies to exclude false (or true) internal initiation. Moreover, stop codon readthrough or frameshifting can be erroneously attributed to reinitiation; so these possibilities should also be excluded. The most appropriate control is to extend the coding region of the uORF far into the downstream ORF by mutating the stop codon of the former in a way to avoid matching reading frames of both ORFs; such a construct should allow zero reinitiation unless the AUG of the first ORF is 'leaky' for initiation, which would still generate some activity from the downstream reporter. Additionally, readthrough can be specifically excluded by inserting an additional stop codon (or a tandem of stop codons) between the translated uORF and the putative reinitiation site, which would reduce readthrough but not reinitiation. Similarly, the possibility of ribosomal frameshifting can be reduced by the insertion or deletion of a nucleotide within the intercistronic region to disrupt the reading frame and eliminate the possibility of frameshifting without affecting reinitiation (unless the nucleotide indel disrupts a cis-acting signal responsible for reinitiation). Alternatively, western blotting could be used to rule out the possibility of either readthrough or frameshifting.

Specific considerations in the assessment of internal initiation. When testing for IRES-dependent translation, the mRNA sequence suspected to promote internal initiation is inserted between two reporters to enable the translation of the downstream reporter. Unlike scenarios involving frameshifting or stop codon readthrough, no fusion product is expected. Therefore, the potential for the translation of the first

cistron to interfere with the proposed IRES must be minimized. This can be achieved by incorporating additional stop codons to prevent any readthrough and by positioning the IRES at a sufficient distance from the first cistron stop codon to ensure that terminating ribosomes do not compromise the structural integrity of the IRES. However, it is important to consider the possibility that integrating an RNA fragment into an unnatural context may affect any potential IRES activity.

Although distant elements affecting stop codon readthrough[114,115] and frameshifting[116] have been reported, it is often the case that only short motifs or structures are examined. Conversely, because internal initiation relies on more elaborate structures, testing longer sequences is essential. Accordingly, all the aforementioned concerns regarding cryptic promoters and splice sites are particularly relevant in the context of IRES testing. It is important to consider that nearly any arbitrarily selected long fragment of a mammalian 5′ untranslated region (UTR) could exhibit cryptic promoter activity, attributable to the abundance of transcription factor binding sites within these regions. Furthermore, a substantial proportion of human genes contain alternative transcription start sites[117], and transcription start site switching can occur during acute stress[118–120]. Some plasmid vectors also contain cryptic promoters upstream of cloned test reporter genes, which can produce unexpected products via alternative splicing[52]. Additionally, long insertions increase the risk of false positives when using in vitro systems like ntRRL, which are not strongly cap dependent.

To reveal cryptic splicing (but not cryptic promoter)-mediated events, the independence of first and second cistron expression can be verified by designing constructs containing a sufficiently long uORF in the 5′ UTR (preferentially overlapping with the main AUG of the first cistron and with the uAUG codon in a strong Kozak context) or by inserting a stable hairpin at the very 5′ end of the transcript to diminish the translation efficiency of the first cistron[62]. However, this approach requires analysis of the transcript level, as the downstream reporter may be affected if such modifications alter the stability of the whole mRNA.

In IRES research, the use of mRNA reporters is distinctly preferable to that of DNA reporters[62]. Using in vitro transcribed reporters eliminates artifacts arising from cryptic promoter activity, unintended splicing or premature transcription termination. When using DNA transfection to evaluate putative IRES activity, promoterless and siRNA-mediated controls (see above) are essential.

Most importantly, however, the bicistronic assay used to examine IRES activity has a notable intrinsic limitation that was not initially apparent. Comparing the expression ratios of upstream and downstream cistrons between bicistronic reporters containing different IRESs is often uninformative. This is because any two long nonspecific arbitrary sequences placed between reporters are highly unlikely to yield equal readouts, while different bona fide IRESs can also exhibit substantially different activities (Fig. 4a). As a result, it becomes challenging to confidently design negative or positive controls for such assays, making conclusions subjective. The comparison can be especially misleading if expressed as a ratio to the negative control rather than to the positive control.

Many putative IRES sequences are derived from natural 5′ UTRs, which, in the case of cellular and many viral mRNAs, are naturally capped and therefore can be bound and scanned by the canonical initiation machinery. Therefore, even if the sequence under investigation suggests internal initiation in a bicistronic context, it is not straightforward to evaluate the contribution of internal initiation to overall translation in its natural context. These complications can be addressed by comparing expression from bicistronic and monocistronic reporters, allowing for an assessment of the relative contributions of different initiation mechanisms (Fig. 4b).

Finally, it is important to note that the term 'IRES' specifically refers to internal ribosome entry rather than cap independence. While the latter arises as a consequence of the former, some mRNAs that definitely lack an IRES and strictly require a free 5′ end can nevertheless be efficiently translated in an uncapped (or capped with the artificial nonfunctional A cap analog) form. In such cases, specific elements known as cap-independent translation enhancers (CITEs) facilitate cap-independent translation[62]. In contrast to IRESs, CITEs cannot direct translation of the second cistron in a bicistronic mRNA (Fig. 4b). Furthermore, the widespread presence of IRESs in the 5′ UTRs of cellular mRNAs is often presumed to be due to the low processivity of the translation initiation complex. However, the processivity is likely to be high[51,121,122]. This has contributed to the perception that any long 5′ UTR must use a noncanonical translation initiation pathway, which does not seem to be the case. Given the considerable proportion of purported 'cellular IRESs' that have been challenged to date[11,44,46,48–52,54,62,65,67–70,111], one should bear in mind that the detected cap-independent activity of a sequence under investigation may easily be attributed to CITEs or even some of the experimental artifacts described above.

MINDR

Based on the caveats described above, we propose the following three minimal reporting requirements that should mitigate many of these issues and should accompany any study with data obtained using dual reporters:

A list of positive and negative controls

As discussed above, the use of appropriate positive and negative controls is critical. However, it may be too impractical to design all the controls described in the previous sections. Nevertheless, the level of confidence in the reported results depends on the specific controls used in an experiment. Therefore, the authors should explicitly describe which positive and negative controls have been used for detecting potential artifacts to help reviewers and readers assess the reliability of the study.

Full sequences of all vectors and inserts

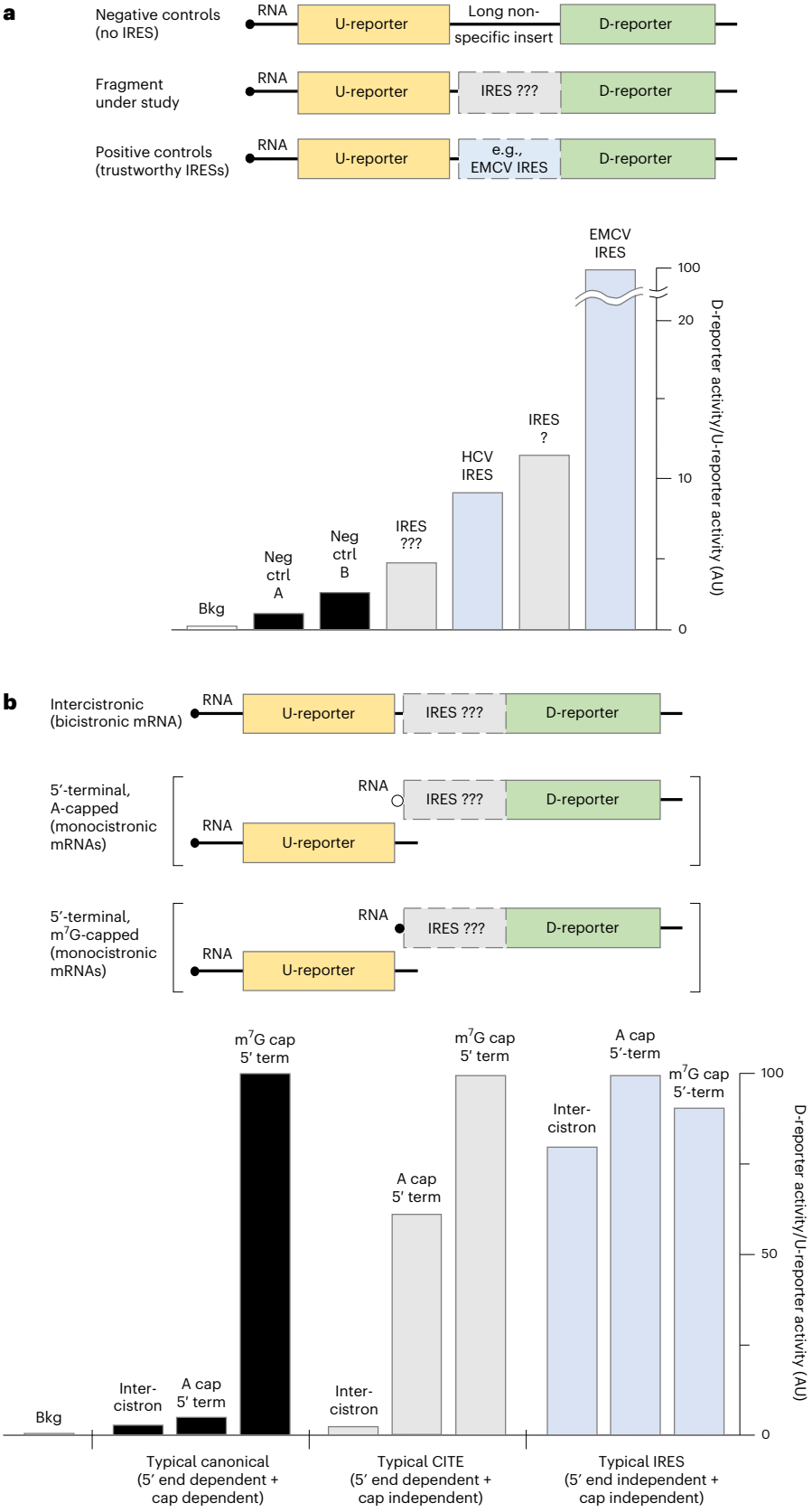
Sequences responsible for potential artifacts, such as transcription enhancers or donor or acceptor splice sites, may be distant from the sequence encoding reporters[52,55]. Therefore, for reproducibility and future interrogation of reported results, the exact sequence of all plasmids used should be provided.

Absolute readout values for each reporter and for the background

The use of dual reporters in general requires the analysis of their ratios rather than absolute values, which are subject to high variability due to technical reasons, such as transfection efficiencies.

Fig. 4 | Challenges of dual-reporter assays in the study of IRES activity. **a**, Simulated results from typical bicistronic assays, inspired by several studies[11,46,50,51,122], and their potential interpretation. Unambiguous conclusions cannot be drawn solely from comparisons of the activities of different bicistronic mRNAs with each other, as control values (both negative and positive) may exhibit substantial variability. IRESs with question marks indicate sequences under study that may (or may not) be considered IRESs with varying degrees of confidence depending on the researcher's preference. AU, arbitrary units; ctrl, control; EMCV, encephalomyocarditis virus; HCV, hepatitis C virus; neg, negative. **b**, A mechanism of ribosome recruitment used by a specific mRNA fragment (for example, a 5′ UTR) can be elucidated through three related dual-reporter assays using the mRNA constructs shown at the top. Three distinct translation initiation mechanisms can be distinguished: canonical cap-dependent scanning, CITE directed or IRES directed, as indicated below. To prepare m^7G-capped transcripts, it is advisable to use CleanCap, anti-reverse cap analogue or post-transcriptional enzymatic capping instead of m^7GpppG, as these methods yield more natural, non-immunogenic and efficiently translated mRNAs. Additionally, ApppG or ApppA can be used to produce transcripts that are stabilized by nonfunctional cap analogs at the 5′ ends. Bottom, typical simulated results as in **a**. Bkg, the background level of reporter activity in mock-transfected cells; term, terminus.

https://doi.org/10.1038/s41594-025-01492-x



Nonetheless, as discussed earlier, consistent differences in absolute values are often indicative of artifacts. Therefore, it is important that, in addition to providing the ratios between the reporter activities, the absolute raw readouts should be made available for each replicate of each construct including background values from control experiments without reporter constructs.

Data availability

No original data have been generated or reanalyzed during the development of these guidelines.

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Acknowledgements

We acknowledge many colleagues who informally supported and encouraged the development of these guidelines. The following authors acknowledge funding support: P.V.B. by the Wellcome Trust (210692/Z/18/]) and Science Foundation Ireland (20/FFP-A/8929); L.S.V. by the Lead Agency (DFG and CSF) (grant 23-08669L) and the Praemium Academiae grant provided by the Czech Academy of Sciences and CZ.02.01.01/00/22_008/0004575 (RNA for therapy by ERDF and MEYS); S.E.D. from the Russian Science Foundation (23-14-00218, the IRES and cell-free sections); C.S.F. by the NIH (R35 GM152137); W.V.G. by the NIH (R01GM132358); Y.C. by the NIH (R01GM130838); J.F.A. by the Irish Research Council (IRCLA/2019/74); D.E.A. by the Russian Science Foundation (20-14-00121, RNA versus DNA transfection section); M. Mariotti by RYC2019-027746-I, PID2020-115122GA-I00 and PID2023-147164NB-I00 by MICIU/AEI (Ministry of Science, Innovation and Universities; State Research Agency of Spain) /10.13039/501100011033 and "ESF Investing in your future.; M.S.S. by the NIH (R01GM148702). J.K. by National Science Centre (UMO-2021/41/B/NZ2/03036). J.S.K. by NIH (R35GM118070). P.L.C. by the NIH (DP1GM146256). S.I. by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) (JP24H02307). M. Mikl by ISF personal grant (2219/22).

Competing interests

G.L. and P.V.B. are cofounders and shareholders of EIRNA Bio Ltd. The remaining authors declare no competing interests.

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Peer review information *Nature Structural & Molecular Biology* thanks Andrei Korostelev and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Primary Handling Editors: Michelle Korda and Dimitris Typas, in collaboration with the *Nature Structural & Molecular Biology* team.

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https://doi.org/10.1038/s41594-025-01492-x

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