



Review

Current Practice in Bicistronic IRES Reporter Use: A Systematic Review

Guus Gijbertus Hubert van den Akker ^{1,†}, Federico Zacchini ^{2,3,†} , Bas Adrianus Catharina Housmans ¹, Laura van der Vloet ¹, Marjolein Maria Johanna Caron ¹ , Lorenzo Montanaro ^{2,3,4,†} and Tim Johannes Maria Welting ^{1,*}, [†]

- ¹ Department of Orthopedic Surgery, Maastricht University, Medical Center+, 6229 ER Maastricht, The Netherlands; g.vandenakker@maastrichtuniversity.nl (G.G.H.v.d.A.); b.housmans@maastrichtuniversity.nl (B.A.C.H.); l.vandervloet@student.maastrichtuniversity.nl (L.v.d.V.); marjolein.caron@maastrichtuniversity.nl (M.M.J.C.)
- ² Department of Experimental, Diagnostic and Specialty Medicine, Bologna University, I-40138 Bologna, Italy; federico.zacchini3@unibo.it (F.Z.); lorenzo.montanaro@unibo.it (L.M.)
- ³ Centro di Ricerca Biomedica Applicata—CRBA, Bologna University, Policlinico di Sant’Orsola, I-40138 Bologna, Italy
- ⁴ Programma Dipartimentale in Medicina di Laboratorio, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Via Albertoni 15, I-40138 Bologna, Italy
- * Correspondence: t.welting@maastrichtuniversity.nl
- † These authors contributed equally to this work.



Citation: van den Akker, G.G.H.; Zacchini, F.; Housmans, B.A.C.; van der Vloet, L.; Caron, M.M.J.; Montanaro, L.; Welting, T.J.M. Current Practice in Bicistronic IRES Reporter Use: A Systematic Review. *Int. J. Mol. Sci.* **2021**, *22*, 5193. <https://doi.org/10.3390/ijms22105193>

Academic Editors: Benoit Chabot and Anne-Catherine Prats

Received: 25 March 2021
Accepted: 12 May 2021
Published: 14 May 2021

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Bicistronic reporter assays have been instrumental for transgene expression, understanding of internal ribosomal entry site (IRES) translation, and identification of novel cap-independent translational elements (CITE). We observed a large methodological variability in the use of bicistronic reporter assays and data presentation or normalization procedures. Therefore, we systematically searched the literature for bicistronic IRES reporter studies and analyzed methodological details, data visualization, and normalization procedures. Two hundred fifty-seven publications were identified using our search strategy (published 1994–2020). Experimental studies on eukaryotic adherent cell systems and the cell-free translation assay were included for further analysis. We evaluated the following methodological details for 176 full text articles: the bicistronic reporter design, the cell line or type, transfection methods, and time point of analyses post-transfection. For the cell-free translation assay, we focused on methods of in vitro transcription, type of translation lysate, and incubation times and assay temperature. Data can be presented in multiple ways: raw data from individual cistrons, a ratio of the two, or fold changes thereof. In addition, many different control experiments have been suggested when studying IRES-mediated translation. In addition, many different normalization and control experiments have been suggested when studying IRES-mediated translation. Therefore, we also categorized and summarized their use. Our unbiased analyses provide a representative overview of bicistronic IRES reporter use. We identified parameters that were reported inconsistently or incompletely, which could hamper data reproduction and interpretation. On the basis of our analyses, we encourage adhering to a number of practices that should improve transparency of bicistronic reporter data presentation and improve methodological descriptions to facilitate data replication.

Keywords: bicistronic reporter; dicistronic reporter; mRNA translation; ribosome; IRES; systematic review

1. Introduction

Reporter gene assays are widely used to study cellular signaling, gene regulation, and mRNA translation. Reporter constructs, in their basic form, consist of a promoter of interest that controls the transcription of a reporter gene, thus allowing a quantitative reading of the transcriptional and translational activity. A plethora of reporter genes is available, ranging

from purely enzymatic, luminescent, to fluorescent [1]. Each has its own advantages and limitations. For example, (radio)enzymatic assays, such as chloramphenicol acetyltransferase (CAT) or β -galactosidase (β Gal) were used initially [2]. Later, luminescent proteins, such as Firefly (Fluc) or Renilla luciferase (Rluc) and their optimized reaction conditions led to the development of faster read-out [3]. More recently, fluorescent proteins, such as green fluorescent protein (GFP; [4]) have allowed for the sorting of specific living cells and other *in vivo* applications [5].

Most reporter assays utilize transient transfection of a cell line. Variation in transfection efficiency or cell number between experimental conditions or replicates can lead to misinterpretation of the results or high variation [6]. Therefore, it has become standard procedure to co-transfect a second constitutively active reporter plasmid to allow correction for such effects. Stable reporter lines have recently gained popularity, for example, in high-throughput screening procedures [7]. The generation of a stable reporter line requires a selectable marker to remove cells that did not incorporate the reporter. This can be achieved by utilizing antibiotic resistance genes or a fluorescent protein for sorting with a separate promoter or in a bicistronic configuration. In addition, some applications demand the possibility of utilizing two reporter genes (e.g., luminescent and fluorescent) at the same time, under the control of a single promoter. This can be achieved with an internal ribosome entry site (IRES), usually from the encephalomyocarditis virus (EMCV), or StopGo sequences, e.g., P2A from porcine teschovirus [8,9]. IRES mediated translation is a distinct mode of protein translation that was first discovered in viruses [10]. The IRES sequence can directly recruit the ribosome or through IRES transacting factors, which is independent of cap-mediated translation initiation, and may not require canonical initiation factors and ribosomal scanning [11]. A database with well-established IRES sequences currently contains >63 viral and >115 cellular IRESs [12].

In the past three decades, our understanding of viral IRES elements has improved significantly [11], and crucial determinants of IRES activity have been uncovered [13]. The use of bicistronic reporters has been instrumental to advance the field of IRES identification, their sequence variation, co-factor identification, and regulation. However, the study of bicistronic mRNA translation is not without potential pitfalls. In a cellular system using plasmid DNA transfections, cryptic splicing and cryptic promoter activity can take place [14]. Control experiments have been designed to rule out such confounding effects. For example, cryptic promoter activity can be evaluated by generating promoterless control plasmids, while cryptic splicing can be assessed with RT-qPCR, Northern blotting, or 5'RACE of reporter mRNA. An even more elegant approach is represented by siRNA targeting of one cistron in combination with RT-PCR [15,16]. Finally, read-through variation between evaluated control and test sequences can distort the results, which was recently shown to be avoidable using 2A StopGo sequences [17]. These 2A-sequences induce a premature stop of protein translation after a specific glycine and re-initiate formation of a new downstream polypeptide encoded at the next codon of the same mRNA. An alternative to plasmid DNA is the transfection of mRNA transcribed outside the cell, which allows for an integrity check by gel electrophoresis. This can also be combined with a cell-free translation assay [18]. A cellular lysate endowed with a high protein synthesis activity, often derived from a rabbit reticulocyte lysate (RRL), is combined with the mRNA in a reaction tube. The addition of specific co-factors to the system or depleting the lysate from endogenous ribosomes and replacing it with isolated ribosomes from sources of interest allows for in-depth ribosome activity studies [18]. Ribosomes can occasionally skip a stop codon, alter reading frames, or re-initiate translation [19]. Dedicated bicistronic reporter assays and analysis methods were developed to quantify such ribosome fidelity aspects [20].

The identification of eukaryotic IRESs in genes crucially involved in stress and apoptosis signaling (e.g., P53 [21]) has led to debates about limitations of bicistronic reporter assays and the definition of an IRES. With the advent of high throughput sequencing coupled to custom libraries, many new 5' and 3' UTR sequence elements (cap-independent translational elements, CITE) have been identified that alter cap-independent translation [22–24].

In addition, it has become apparent that the ribosome itself is not a static player in protein synthesis [25]. The protein composition or ribosomal RNA (rRNA) post-transcriptional modifications (PTM) affect its function [26,27]. Novel techniques to quantify small changes in rRNA PTM open up new possibilities for epitranscriptomics [28,29], which are expected to become increasingly complex when more types of RNA PTMs can be accurately quantified [30,31]. New insights in ribosome heterogeneity in stem cell differentiation [32], human diseases related to single gene defects (ribosomopathies [33]), or complex multifactorial diseases such as cancer [34] underline the importance of robust reporter assays to interrogate ribosome function. As such, the ribosome is now considered a drugable target for human disease [35,36].

In this review, we aimed to provide a focused and representative example of bicistronic IRES reporter use. For this purpose, we performed a systematic literature review to provide an unbiased overview of current practice in methodology, control experiments, and data reporting. Important inclusion criteria for further analysis were experimental studies using an adherent eukaryotic cell system or cell-free translation. We focused on technical and methodological aspects of experiments, as well as data visualization, control experiments, and normalization. Finally, we provide some basic recommendations for the use of bicistronic reporter assays that should benefit all users of bicistronic reporters. This is expected to contribute to better reproducibility of data and facilitate comparison of results between studies.

2. Results

We selected 176 studies for evaluation of reporter system methodology, data representation, and normalization (Figure 1, Table 1 and Table S1, [37–212]). From these studies, 41 (23%) utilized a cell-free translation system. Relevant design parameters of a bicistronic reporter construct are summarized in Figure 2A. First, we evaluated the different types of reporter genes used over time (Figure 2B). In total, we identified 45 different reporter configurations. From 1994 until 2000, reporters based on luciferase and CAT were the most frequently used. The Fluc/Rluc luciferase system was by far the most popular from 2000 until the present. The use of autoradiography for cell-free translation assays was prevalent between 1994 and 2005. Fluorescent reporters acquired a modest but stable popularity from 2000 onwards.

Table 1. Overview of information extracted from the 176 included studies.

Collected Information	In Vivo Translation	In Vitro Translation
PubMed ID, authors and title	Y	Y
Year of publication	Y	Y
The plasmid backbone name(s)	Y	Y
The bicistronic reporter gene configuration	Y	Y
Monocistronic reporter use (yes/no)	Y	Y
The name of the investigated IRES or 5' UTR	Y	Y
The number of iress or 5' UTRs studied in one article	Y	Y
The name of used cell line(s) or primary cell type	Y	NA
The number of cell lines used in one article	Y	NA
The name of the transfection reagent (if applicable)	Y	NA
Reported cell density (yes/no, and if yes, what was mentioned)	Y	NA
Time post-transfection/translation initiation of the measurement	Y	Y
Method of reporter protein detection	Y	Y
In vitro transcription system	NA	Y
mRNA isolation method	NA	Y
mRNA amount	NA	Y
Lysate type (source of ribosome preparation)	NA	Y

Table 1. Cont.

Collected Information	In Vivo Translation	In Vitro Translation
Temperature of reaction °C	NA	Y
Co-factors (when reported)	NA	Y
Data presentation categories	In vivo translation	In vitro translation
Ratio (raw)	Y	Y
Ratio (normalized)	Y	Y
Ratio and cistrons separately	Y	Y
Cistrons separately	Y	Y
Raw data available (RLU/RFU/CPM)	Y	Y
Normalization and control categories	In vivo translation	In vitro translation
Correction for empty reporter or IRES positive control	Y	Y
Correction for control condition (same reporter, non-treated cells)	Y	Y
Correction for total protein levels	Y	Y
Correction for reporter mRNA levels	Y	Y
Background of non-transfected cells shown	Y	NA
Background subtraction/correction	Y	NA

The table is divided in three parts: collected information, data presentation, and normalization/controls (indicated in bold). Y (yes) or not applicable (NA) indicates whether a parameter applies to in vivo or in vitro translation assays.

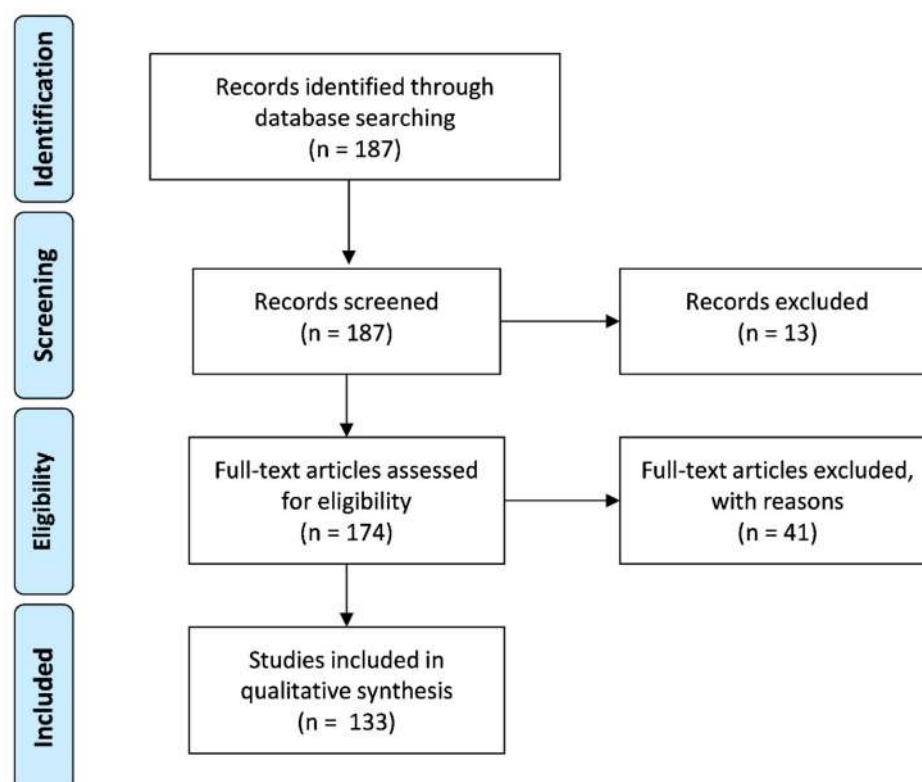


Figure 1. PRISMA flow diagram of eligible studies for this systematic review. Two hundred and fifty-seven studies were identified and screened. Twenty-eight studies were excluded after screening the title and abstract. Fifty-three additional studies were excluded on the basis of violation of inclusion criteria. A total of 176 full text articles were included in this study. The identity of all 257 articles and the 176 included studies can be found in Table S1.

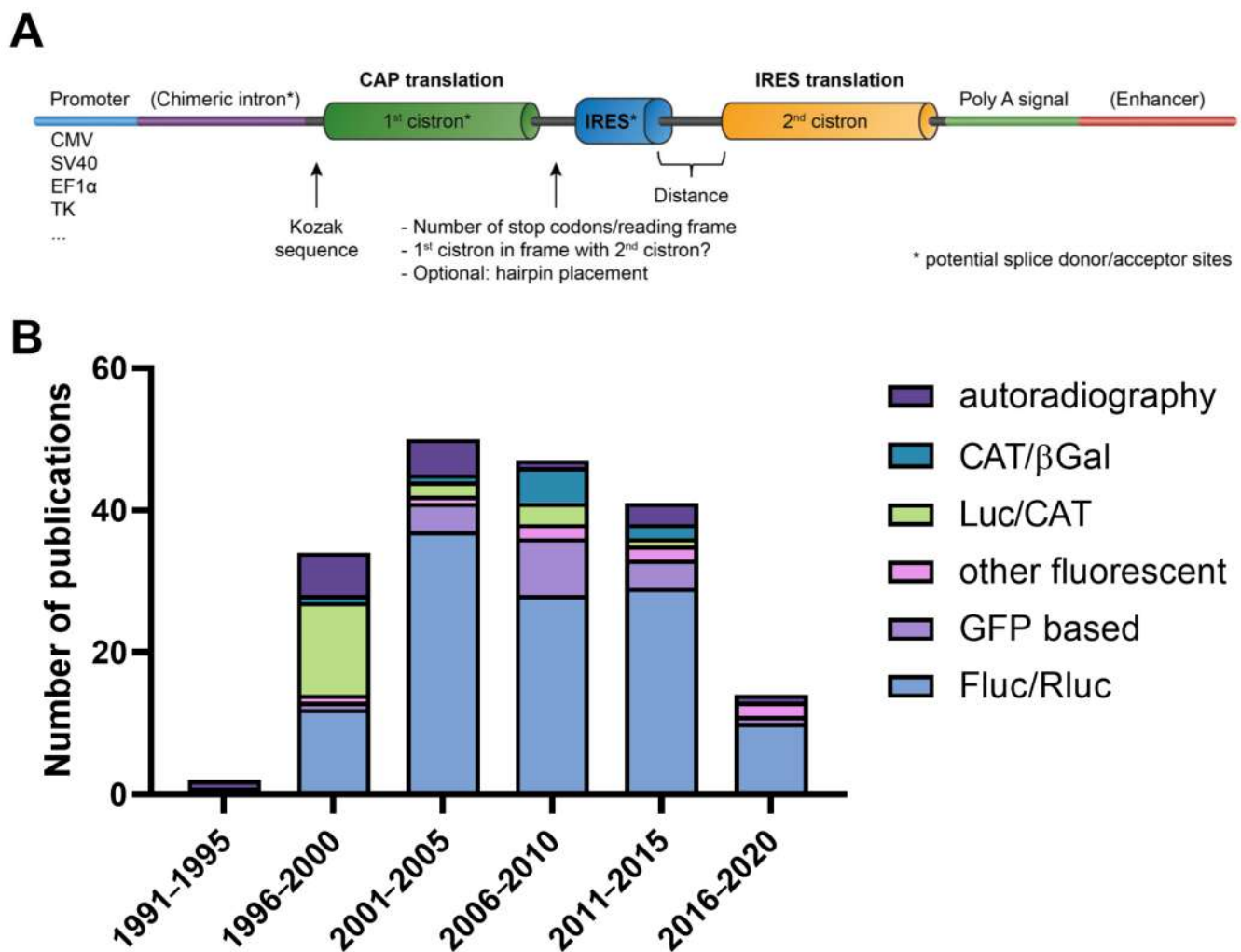


Figure 2. Bicistronic reporter design considerations and reporter gene use over time. (A) Relevant reporter design parameters are depicted. The choice of promoter and use of a chimeric intron and/or enhancer alters the mRNA expression levels, stability, and translation efficiency. The reporter genes themselves, as well as their positioning, can influence the observed IRES activity. The exact IRES sequence, for example EMCV sequence variants, can also have large effects. Potential read-through can be inhibited with (multiple) stop codons behind the first cistron (in all three reading frames) or inclusion of an inhibitory hairpin (second arrow). The first cistron always contains a Kozak sequence, but in some specific plasmid backbones, we identified a second Kozak sequence at the second cistron. A common criticism on the bicistronic reporter assay is the possibility of cryptic splicing due to specific reporter element (*) or cryptic promoter activity of the IRES sequence that can lead to false positive results. The former can be evaluated with Northern blot, RT-PCR, or 5'-RACE and ruled out with a monocistronic reporters or a cell-free translation system; the latter can be ruled out by using promoterless control constructs or a cell-free translation system. Cryptic promoter activity can be reduced with SPA sites, although this can lead new cryptic splicing events. (B) Reporter gene configuration was summarized by allocation to six functional groups in five-year bins. The first publication was from 1994, which explained the low number of publications in the first bin. β Gal = β -galactosidase, CAT = chloramphenicol acetyl transferase, Luc = any luminescent protein, GFP = green fluorescent protein, Fluc = Firefly luciferase, Rluc = Renilla luciferase.

2.1. Critical Determinants of Bicistronic Reporter Plasmid Design and IRES Activity

Details on plasmid backbones were difficult to differentiate since the origin of the bicistronic reporter plasmids was not always traceable. In addition, modifications of various vectors (e.g., psiCHECKv2) to generate a bicistronic Fluc/Rluc reporter were often named pRF. As such, there is a large variety in the employed vector backbones (111 unique names) with limited traceability (Figure 3A, left panel). Aside from the CMV promoter, the SV40, HSV TK, PGK, and EIF1 α promoters are often used to drive

transcription of the bicistronic reporter, although we did not quantify the frequency of their use. Interestingly, a direct comparison of the same bicistronic EMCV IRES construct with either the CMV or PGK promoter exhibited a similar cap-mediated translation but a higher IRES-mediated translation with the PGK promoter [135]. A second study demonstrated higher IgG production with the EIF1 α promoter when compared to CMV in a bicistronic vector, but this difference disappeared when swapping the cistron position [126]. The positioning of Fluc, Rluc, and CAT cistrons is known to affect IRES activity [73]. In terms of reporter types, the XIAP 5' UTR was evaluated side by side in a Fluc/Rluc and a CAT/ β Gal system [109]. It was concluded that the CAT/ β Gal system appeared to be more reliable. The CMV promoter and intron A are known to contain a splice donor site [213]. The combination with an acceptor splice site within the coding sequence of Rluc and/or the 5' UTR under investigation can lead to aberrant splicing and misinterpretation of cap-independent translational activity. Of note, it was shown that the bacterial pUC and pMB1 origin of replication sequences induced cryptic splicing and promoter activity in pGL3/pGL4 plasmids that can lead to false positive IRES activity [169]. Transcriptional pause sites (SPA) were introduced to counteract cryptic promoter activity, but this did not abrogate the cryptic splicing effects. The 3' SV40 translational enhancer, which is present in pGL3-based Rluc/Fluc reporter constructs, was shown to mediate second cistron translation by cryptic promoter activity [114]. Transfection of in vitro-transcribed mRNAs from the same plasmid showed that cellular IRES translation (HIF-1 α , VEGF, C-MYC, XIAP, VEGFR-1, or Egr-1) was lower than suggested by plasmid DNA transfection [114,214]. Therefore, it has been hypothesized that a nuclear event is required for efficient cellular IRES translation [214,215]. For example, HNRNPM and NONO were implicated in FGF1 IRES activity [216]. Another parameter that was evaluated in the literature is the number of nucleotides that are used as a spacer between an IRES element and the second cistron. An optimum of \approx 30 nucleotides spacer sequence was established for the HTLV-1 and poliovirus IRESs [50]. In a similar study, increased spacer length (43–89 nt) decreased FMDV IRES activity [45]. In addition, the presence of 1–5 alternative start codons (in or out of frame) and the potential of hairpin formation in the spacer sequence reduced IRES activity. The ideal flanking sequence of the translation start codon was evaluated for the FMDV IRES and strongly affected eGFP protein expression [204]. Studies reporting on direct comparison of dual luciferase with dual fluorescence reporters were rare. At least for multiple HCV IRES sequence mutants both reporter configurations provided identical results in HEK293 and Huh-7 cells [164]. In the same study, stimulation of cells with IFN γ for 36 h demonstrated a higher sensitivity of the dual luciferase system in comparison to the fluorescent reporter. Two rare reporter gene configurations were used to enhance the signal from the first or second cistron. A 9 nt sequence derived from the Gtx 5' UTR, which is complementary to a region of 18S rRNA, could strongly enhance second cistron translation [57]. Ten copies of this 9 nt sequence without spacers was 10-fold more active in a bicistronic Luc/CAT reporter than the EMCV IRES. In a different study, the signal of the reporter gene (Rluc or eGFP) was amplified by a positive feedback loop utilizing IRES-mediated translation of the yeast activator Gal4 and herpes simplex virus VP16 fusion protein (Gal4/VP16) that acts as a transcription factor [217] and Gal4 upstream activation sequences [113]. Next, we evaluated the employed IRESs. The total number of unique IRESs or 5' UTRs in 176 studies was 116. Around one-quarter of the studies (23%) included the EMCV IRES, closely followed by the HCV IRES (14%; Figure 3A, middle panel). The first eukaryotic IRES in this list was P53 (1.0%). Although the majority of publications used a single IRES (56%), 40% of the publications used 2 to 4 different IRESs, which excluded control constructs (Figure 3A, right panel). In conclusion, there are many important design parameters for a bicistronic reporter that affect its expression level. Because IRES bicistronic reporters have both been used and studied intensely, there is a large body of knowledge with various reporter gene configurations.

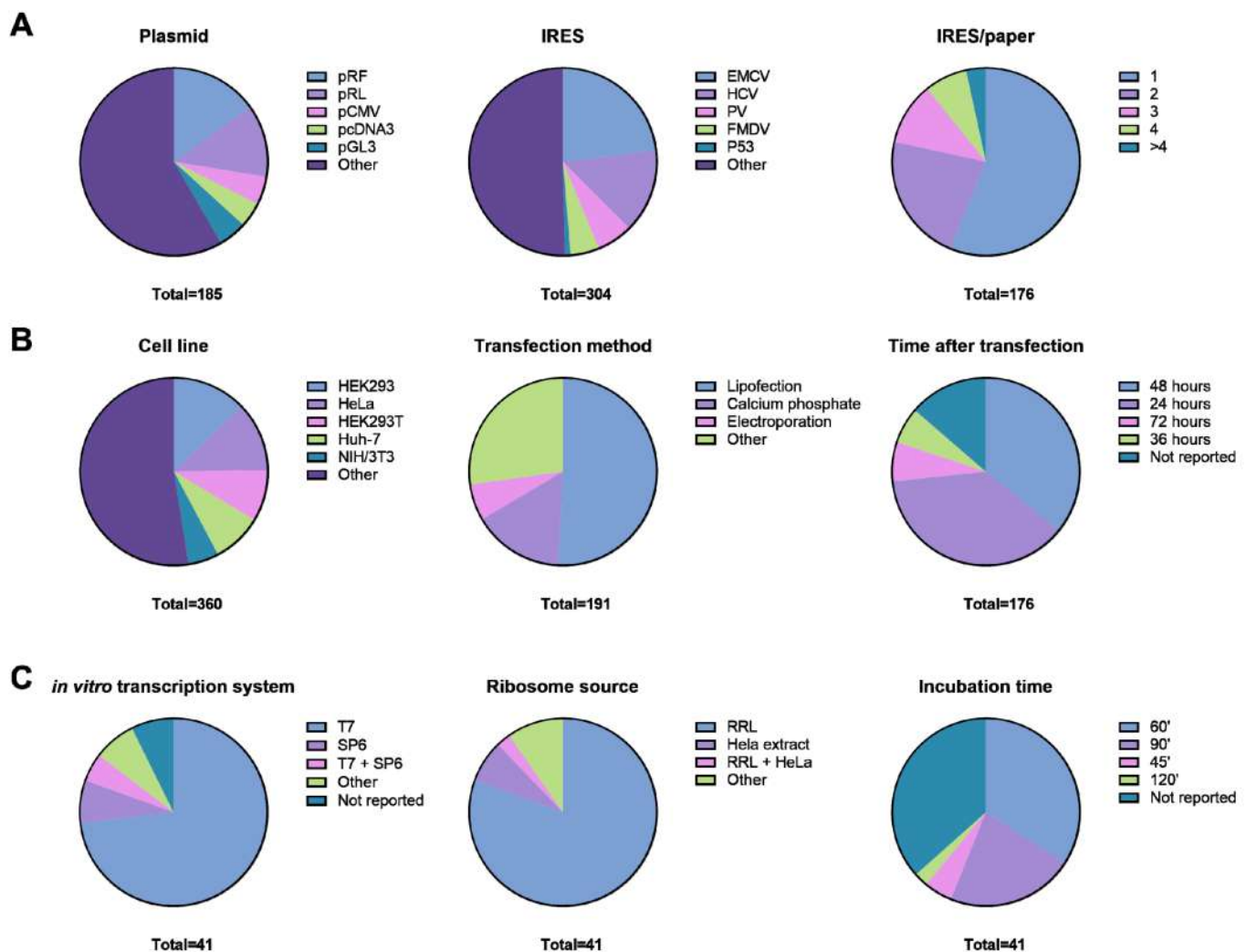


Figure 3. An overview of extracted information from included studies. (A) Plasmid, IRES, and the number of IRESs/publication were quantified and depicted in pie charts. (B) Cellular translation assay parameters. Transfection methods were combined in four categories for easier visualization. Time of transfection refers to the measurement time point of the reporter post-transfection. (C) Cell-free translation assay parameters. Note: total numbers do not always match the number of included studies due to the use of multiple parameters in one study.

2.2. The Most Commonly Used Cell Lines and Cell Type-Specific IRES Activity

Cell lines were transiently transfected with reporter plasmids in nearly all studies, and few studies performed mRNA transfections. We focused on the following experimental details in these publications, amongst others: cell line(s), transfection reagent, and timing of reporter measurements (Table 1). The number of different cell lines [163] used in the analyzed publications was larger than the number of IRESs or 5' UTRs (Figure 3B, left panel). Ninety-two studies used more than one cell line. The most popular cell lines were HEK293 (13%), HeLa (12%), HEK293T (9%), Huh-7 (8.6%) cells, and NIH/3T3 (5.3%), presumably because some of these cell lines are known to be easy to transfect. The high frequency of Huh-7 cell line use is due to the extensive study of the hepatitis C virus in this liver cell line and therefore also the HCV IRES. Other well-known cell lines that are often employed were HEPH2 (4.7%), Cos7 (3.4%), and MCF7 (3.0%). Only six studies utilized primary cells, such as mouse embryonic stem cells, embryonic fibroblasts, skin fibroblasts, neurons, or bone marrow-derived stromal cells [58,64,75,76,161,163]. A couple of studies stood out due to multiple comparisons between five or more cell lines and three or more viral IRESs [131,137,149,150,161,171,198]. In these studies, a certain cell line had an intrinsically high or low IRES activity, independent of the actual IRES under investigation.

A notable exception was a comparative study of the FGF2 and EMCV IRES in 14 different cell lines [58]. FGF2 IRES activity was reported to be variable between cell lines, while the EMCV IRES activity was constant. GAL-4 5' UTR activity was very similar to the cMyc IRES in five cell-lines [131]. Variants of the HAV IRES showed higher activity in the hepatocytic cell lines HepG2, Huh-7, and HLE, which was not the case in three non-hepatocytic cell lines: HeLa, BSC-1, and CV-1 [157]. Finally, hTPH2-5' UTR variants showed variable reporter expression in six different cell lines, which was shown to be caused by cryptic promoter activity [146]. In summary, many different cell lines have been investigated with bicistronic IRES reporters. Since the number of different IRESs and cell lines is high, there are many unique combinations between IRESs and cell lines in the literature. However, comparative studies seem to indicate that this is less troublesome for data generalization than expected.

2.3. Methods for Transgene Expression and mRNA Translation In Vivo

Plasmid DNA or mRNA can be transiently transfected using various methods. Calcium phosphate transfection has been used for the longest time [218]. However, not all cell lines can be efficiently transfected with this method. Therefore, alternatives have been developed, such as electroporation [219], lipofection [220], and polymers [221]. We identified 39 different transfection reagents, of which Fugene 6 (17%) was the most used, closely followed by Lipofectamine 2000 (16%) and calcium phosphate (16%). A multitude of variants of Lipofectamine 2000, including Lipofectamin (+) and Lipofectin made lipofection the most frequently used transfection method (51%; Figure 3B, middle panel). Calcium phosphate was frequently used as well at 16%, and electroporation was used in 6% of the studies. Protocol parameters can affect the signal of specific cistrons. For example, increasing amounts of transfected plasmid DNA (10 ng to 5 µg) increased the cap-mediated signal without affecting the EMCV IRES signal [52]. Thus, the amount of plasmid DNA and consequently the mRNA levels can influence the observed IRES activity ratio. The use of viral reporter systems was rare in our dataset [71,76,121,147,149,172,178,188]. In retro-, lenti-, or adeno-associated virus constructs, the IRES itself was used to drive translation of a transgene. In viral reporter studies, the bicistronic IRES design was often directly compared to other reporter configurations (monocistronic, 2A StopGo sequence, etc.), usually for flow cytometry applications. A dose of lentivirus equivalent to 100 ng p24/24 wells effectively provided the most optimal luciferase signal in four different cell lines [149]. In one study, a tetracyclin-inducible IRES-GFP construct was generated and it was reported that aberrant splicing, aberrant polyadenylation, or fusion proteins could be generated [172]. Importantly, these issues depended on specific first cistron coding sequences. Overall, transfection protocol-related methodology is often reported with "according to manufacturer's recommendations". Relatively few studies address the effect of transfection reagents or protocol variables on bicistronic reporter activity.

2.4. Temporal Effects of Bicistronic Gene Transcription and mRNA Translation In Vivo

An important aspect of bicistronic reporter use is the time after transfection that is used for the measurement. The build-up, stability, and degradation of the reporter plasmid DNA, mRNA, and proteins affect the measured signal. Translation and/or stability of a reporter protein differs between reporter configurations, e.g., fluorescent proteins are known to require a longer maturation time to allow folding when compared to luciferases [222]. The most frequently used measurement time point after transfection was 24 or 48 h post-transfection with 38% and 36%, respectively. Less prevalent was 72 and 36 h with 6.8% and 6.3%, respectively (Figure 3B, right panel). The timing of measurements after transfection of the reporter was not reported in 14% of the cases. Nineteen studies measured at multiple time points after transfection. From time series experiments, it was shown that for plasmid DNA, the optimal time point to detect differences lies between 36 and 48 h post-transfection [205]. For the EMCV and HRV IRES, maximum activity in a Fluc/Rluc configuration could already be achieved at 12 h post-transfection [115]. Importantly, the

exact sequence of the EMCV IRES in the construct had a large effect on the signal. For mRNA transfection, the largest differences were reported after 24 h in a time series experiment [160]. Studies that investigated multiple time points were often primarily focused on the effect of an IRES on viral replication [132,187]. In a few studies, the effect of a stimulus on IRES activity was measured as a function of time. In a 4–12 h hypothermia time series (30 vs. 37 °C), a consistent upregulation of viral IRES activity was found within 4 h that peaked at 12 h (equivalent to 36 h post-transfection) [198]. This was similar to a recent study with the ACC1 5' UTR, where tunicamycin and thapsigargin induced peak IRES activity at 12 and 3 h, respectively [207]. HCV IRES activity was induced by hydrogen peroxide within 4 h in a 1–8 h time series [118]. A differential effect of IFN α on HAV IRES activity after 36 h was demonstrated in Huh-7 cells (down) and C13-3 cells (up), without an effect on cell viability [110]. The regulation of IRES-mediated translation over longer time periods has been minimally investigated. One study demonstrated differential use of the *Runx2* IRES in MC3T3-E1 over 14 days of differentiation using a stable transfection [101]. This IRES activity partially controlled Runx2 isoform expression. An in vivo knock-in mouse of the Utrophin (Utrn) IRES with CAT and β Gal cistrons was generated to demonstrate tissue-specific activity of this IRES in skeletal muscle [158]. A Fluc/Rluc reporter containing the Fgf2 IRES was knocked-in to demonstrate differential effects of hyperglycemia depending on tissue type [103]. In conclusion, few studies address temporal regulation of IRES activity. In some studies, the time point of the measurement was not reported, which can hinder replication of the study. However, time series experiments seem to indicate that an increase or decrease in IRES activity can be measured over a broad time period. The ideal time point for measurement may differ between IRESs, and it was previously recommended to routinely perform time series experiments [6].

2.5. The Use of the Cell-Free Translation System

The cell-free translation system was used to test IRES activity and in most cases for comparison with the in vivo system. In addition, it was used to exclude potential cryptic splicing or promoter activity [154,156] or to investigate the effect of reaction parameters on translational susceptibility [174,201]. This assay was used in 41 of the included studies (23% of total), of which 10 studies utilized monocistronic reporters. The type of bicistronic reporter was primarily the Fluc/Rluc type with 55%, followed by Luc/CAT (29%). The in vitro transcription system to generate mRNAs was predominantly T7 RNA polymerase-based (73%; Figure 3C, left panel), while the used cell-free system was usually a rabbit reticulocyte lysate (80%; Figure 3C, middle panel). The incubation times for protein translation varied from 15 to 120 min, where 60 min was the most frequent (34%), and this incubation period was not reported in 37% of the studies (Figure 3C, right panel). The reaction temperature was 30 °C in 54% of the studies and 37 °C in 7% of the studies, and not reported in 34% of the cases. RRL samples are almost exclusively incubated at 30 °C and not at the physiological body temperature of a rabbit (≥ 37 °C; [223]) due to the observation that 37 °C incubation led to a premature stop of protein synthesis [224]. Of note, this problem was not reported with HeLa lysates that are incubated at 37 °C [77,107,201,225]. Certain parameters that we intended to evaluate were highly variable and not consistently reported, such as co-factors in the translation reaction (cap analogue, etc.), mRNA amount (moles, mass, concentrations), and purification method of the mRNA (gel extraction, phenol/chloroform, spin columns, or LiCl precipitation). None of the included studies using cellular IRESs reported the addition of cellular extracts to the RRL system. This was previously done to overcome the poor translation efficiency of entero and rhinoviral IRESs [226] and might be crucial for translation initiation of cellular IRESs. Of note, the ability of polypyrimidine tract-binding proteins to act as IRES transacting factors was demonstrated by adding them directly to the RRL [152]. In one study, mRNA concentration effects were investigated using MDV IRES mRNAs and a control mRNA not containing the IRES [153]. A strong increase in IRES activity was observed between 0.4 and 1.6 μ M mRNA. Autoradiographical detection of signal with 35 S-methionine and SDS-PAGE was

more often used than bioluminescence (57% vs. 43%), although the latter has become more frequently used over the past 15 years. Importantly, the validation of cellular bicistronic reporter activity with a cell-free translation assay can reveal false-positive IRES activity in the bicistronic reporter related to cryptic promoter activity [146]. However, not all experimental conditions are consistently reported.

2.6. Visualization of Bicistronic Reporter Data: Ratio vs. Separate Cistrons

There are multiple ways to visualize and report bicistronic reporter data (Figure 4A). The activity ratio between the second and first cistron is often used for IRES bicistronic reporters, either as a ratio of the measurements or as a fold change (Figure 4B; white vs. gray bars).

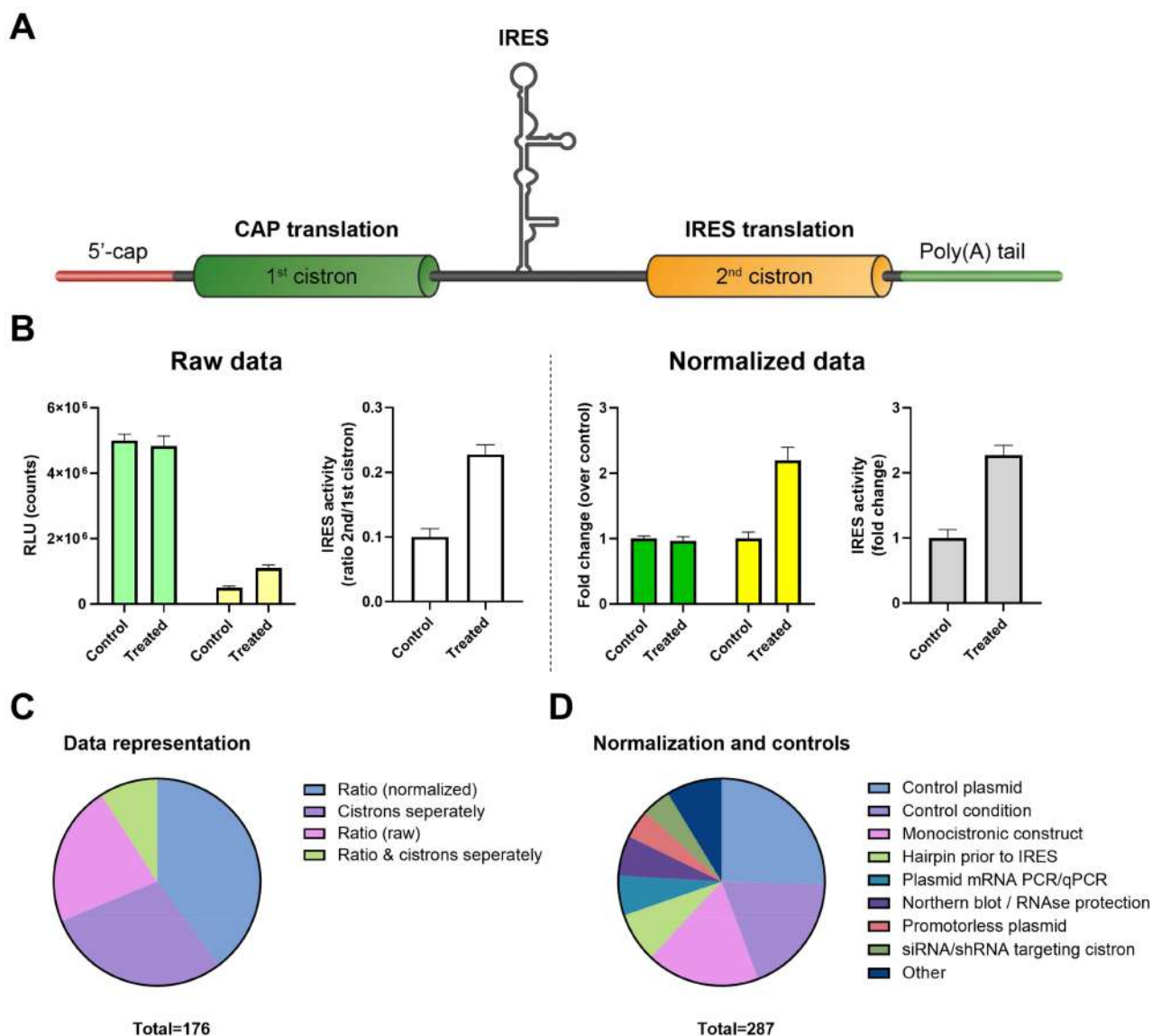


Figure 4. Data representation possibilities and overview. (A) Schematic of a bicistronic IRES reporter mRNA. (B) Visualization options for bicistronic data. Left: raw data from separate cistrons or the calculated ratio. Right: normalized cistrons separately and a normalized ratio. The latter is easier to interpret, however, relevant information is also lost about the measurement and relative signal differences. (C) Data representation in analyzed publications. (D) Normalization strategies and control use. Note that multiple normalization strategies or controls were combined in a large number of studies. The prevalence of the top eight normalization or control methods is depicted in the pie chart.

Data from the individual cistrons can also be presented (Figure 4B: dark green/yellow bars). This provides additional information about what cistron was regulated when compared to reporting the ratio alone. The measured luminescence or fluorescence values can be provided in a table or graph to improve transparency (Figure 4B: light green/yellow bars). We analyzed the representation of the data to obtain an overview of the preferred strategies for data presentation (Figure 4C). The majority of studies showed the ratio between both cistrons, which was either the normalized ratio (40%) or actual ratio (22%). In 29% of the studies, only the activity of the individual cistrons was shown, which reflects the actual measurements. Only 9% of the studies showed both the ratio and individual cistron data. Of note, one-third of the articles showed the raw data (RLU for luciferase, CAT/ β Gal activity, or fluorescence intensity). Overall, reporting the ratio in combination with separate cistrons appears to be the most informative, preferably with a reference to the raw data in the figure legend or (supplementary) table when fold changes are graphed.

2.7. Normalization and Validation of Bicistronic Reporter Data

Normalization strategies are utilized to remove the potential influence of changes in cap-mediated translation, cell number, differences in transfection efficiency, or transcriptional activity, all of which can induce undesirable skewing of data in cellular assays. For example, cap-mediated Rluc signal can be normalized to *Rluc* mRNA copy number, Rluc plasmid copy number, β galactosidase activity, or total protein levels. Most of these parameters, which vary inherently in a cellular system, can be well-controlled in cell-free translation assays. Secondly, there are clear differences between the type of study and the controls they employ in the literature. IRES identification articles tend to use many different types of plasmid controls, for example, inclusion of hairpins between cistrons, monocistronic and promoterless variants, and mutations or truncations of the 5' UTR/IRES. Articles that utilize an established IRES often lack these types of control plasmids, although an empty plasmid backbone can be used. A single control was used in 24% of the studies, the majority used 2–3 controls (63%), and ≥ 4 controls was less frequent (13%). The most frequently used type of control was an empty control plasmid and sometimes a highly active IRES for reference (25%; Figure 4D). In 19% of the cases, a control condition with the same IRES construct, for example unstimulated or non-treated cells, was used. Monocistronic constructs to validate findings were used in 18% of the cases. Inclusion of a hairpin prior to the IRES (7.7%), plasmid-derived mRNA analyses or Northern blot, and RNase protection assays (6.3% each) were also used quite often. Controls that were less frequently used are a promoterless control vector (4.5%), siRNA or shRNA targeting of cistrons (4.6%), cell viability test (MTT, XTT, and others; 2.4%), an indication of non-transfected cell background level or correction (2.1%), normalization to total protein levels in a lysate (1.4%), comparison of capped and uncapped in vitro-transcribed RNA (0.7%), a swap in the orientation of the IRES from 5'-3' to 3'-5' (1.4%), or plasmid DNA level correction (0.7%). In summary, a multitude of controls can be used to validate identification of a new IRES to prevent incorrect interpretation of the bicistronic reporter data. For established IRESs, normalization controls are still useful. The use of an empty control plasmid appeared to be a straightforward, frequently used, and informative control condition.

3. Discussion

In this systematic review, we characterized in an unbiased manner how the bicistronic reporter assay is used from a methodological perspective. We focused on IRES-mediated translation since this is the most frequently used, sensitive, and critical application for this type of reporter. A plethora of technical studies covering plasmid characteristics, sequence variation, and undesirable cryptic splicing or promoter activity has been conducted in the past three decades. New developments in the field have led to the discovery of a multitude of cap-independent translational elements [23] and new evidence for specialized ribosomes [227], which ultimately requires stringent validation using this type of reporter.

Although we analyzed a significant number of articles, it should be noted that we did not identify and include some well-known and highly cited articles in the IRES field [228–231]. This result could not be avoided, since analyzing all articles on IRESs would be an insurmountable task. Despite this limitation, we obtained a significant representation on the use and reporting of bicistronic IRES reporters. Ultimately, this should lead to more transparency and easier reproducibility of published findings. Naturally, many different studies were analyzed, with different research objectives, such as viral IRES mechanistic studies, cellular IRES identification studies, cellular IRES regulation studies, and studies where a bicistronic reporter with an IRES was used. However, this review should provide a comprehensive overview of the critical assay parameters that have been evaluated in a systematic manner for all potential users.

The most used bicistronic reporter configuration was the Fluc/Rluc reporter gene combination. This is likely related to the high sensitivity, linearity of the signal, similar maturation times, and proteolytic stability of these luciferases [232]. Although CAT/ β Gal reporters see limited use in recent times, their susceptibility to cryptic splicing was shown to be lower than Fluc/Rluc reporters [109]. Fluorescent reporters can be used for flow cytometric applications; however, reporter protein maturation times are substantially longer [222], and therefore sensitivity was demonstrated to be lower [164]. To our knowledge, newer luciferases, such as NanoLuc derived from *Oplophorus gracilirostris* [233], have not yet been used in bicistronic reporter configurations. Of note, for dual expression of GFP and RFP, it was shown that a 2A StopGo sequence showed better co-expression characteristics than the EMCV IRES [210]. This is likely related to the different mode of translation. Therefore, if the sole objective is to co-express two cistrons from one mRNA, 2A StopGo sequences should be seriously considered.

In general, it was challenging to deduce the exact reporter construct composition, due to extensive back referencing and limited availability of sequences and bicistronic IRES reporter constructs in literature and plasmid repositories. For example, the search terms used for this study only identified two Fluc/Rluc IRES reporter plasmids at the Addgene repository (#45642 and #18673), while we found over 111 unique plasmid names in the literature. Many research groups generated their own bicistronic Fluc/Rluc reporter that often received the same abbreviation (pRF), which adds to potential confusion. With current next-generation sequencing techniques, we recommend making plasmid backbones and IRES sequences available upon publication. A nice example is the development of a dual luciferase reporter backbone with flanking 2A StopGo sequences to prevent read through (Addgene #119760, [17]), although this vector has not yet been used to evaluate IRES mediated translation.

The EMCV IRES was the most used in the analyzed literature, presumably due to its high activity. However, we also found the use of a large number of different IRESs or 5' UTRs in an even higher number of different cell lines. This implies that many findings have been obtained using a specific IRES or 5' UTR in a specific cell line that have not been replicated by an independent group. As a result, there is limited data about cell line or cell type-specific activity of one IRES or 5' UTR. This cell type specificity is highly relevant in the context of ribosome heterogeneity and cellular differentiation or disease-specific mRNA translation studies [32,34].

The most commonly used transfection method was lipofection, while stable reporter cell line use is relatively rare. The latter is presumably related to the continuous mRNA expression of such a stable transgene, where reporter protein turnover becomes a factor. An inducible system might be an elegant solution that could facilitate temporal regulation studies. Reporting of relevant transfection methodology is usually done, and it is recommended to at least specify the amount of transfected DNA/RNA, number of cells, confluency, and incubation times. In cell-free translation systems, the lack of reporting such parameters could be motivated by the fact that commercial assays and protocols are often used. Measurement time points post-transfection were usually between 24 and 48 h. For specific treatments (hypothermia, hydrogen peroxide, and IFN α), alterations in IRES

translation were observed within 4 h post-stimulation, and peak inductions were observed at later time points up until 12 to 36 h. This is consistent with the notion that translational regulation is a potent mechanism to modify protein expression [234]. Transgenic knock-in mice provided evidence for cell type-specific activity of the Fgf2 IRES and the Utrn 5' UTR [58,103,158]. Temporal regulation of eukaryotic IRES or 5' UTR translation in the context of environmental changes is currently understudied since most publications have focused on identifying new IRESs or CITEs. In other study types, the EMCV IRES, which was shown to provide stable second cistron expression in many cell lines and cell types [58], was used to translate the second cistron. In cell-free translation systems, the most used lysate source for translation is the well-known RRL, despite the known problems associated with this system [6]. This is probably due to its wide diffusion, commercial availability, and the lack of valid commercial alternatives. However, the use of cell-free translation systems, in particular RRL, can lead to low translation efficiency in the study of some cellular IRESs that require a “nuclear experience” [214,215,235]. For these cellular IRESs, the availability of particular nuclear IRES transacting factors is thought to be required for their proper translation initiation. For this reason, RRL is sometimes supplemented with ribosomal salt-wash fractions from cell extract [236], but none of the included articles used these co-factors. The use of bicistronic reporters in cell-free assays is complex and only used by a specialized minority of researchers. Despite this, the considerations and problems encountered are in part similar to those of *in vivo* assays, although cryptic promoter activity or undesired splicing problems are avoided.

Data presentation of bicistronic reporters can be performed in multiple ways. In the pre-2000 era, we found a majority of articles that showed only data of individual cistrons. Presenting the ratio of both cistrons alone is a more modern development that is less transparent and might be misleading. In an ideal IRES study, cap-mediated translation would remain equal as a result of a treatment compared to control and the IRES-mediated translation would be induced (Figure 5A). However, a decrease in cap-mediated translation and equal IRES-mediated translation could result in a similar IRES activity ratio change (Figure 5B). This last example can also lead to misinterpretation if the observed IRES activity is close to background levels: IRES signal will remain the same, while cap signal is reduced, leading to the false-positive conclusion that IRES activity is increased. Cap and IRES activity can both be negatively affected by a treatment, such as apoptosis induction [237] or amino acid starvation [238]. Reporting the ratio in combination with presenting data from the separate cistrons appears to be the most informative way of presenting these kinds of data, preferably with a reference to the raw data and background levels when fold changes are graphed.

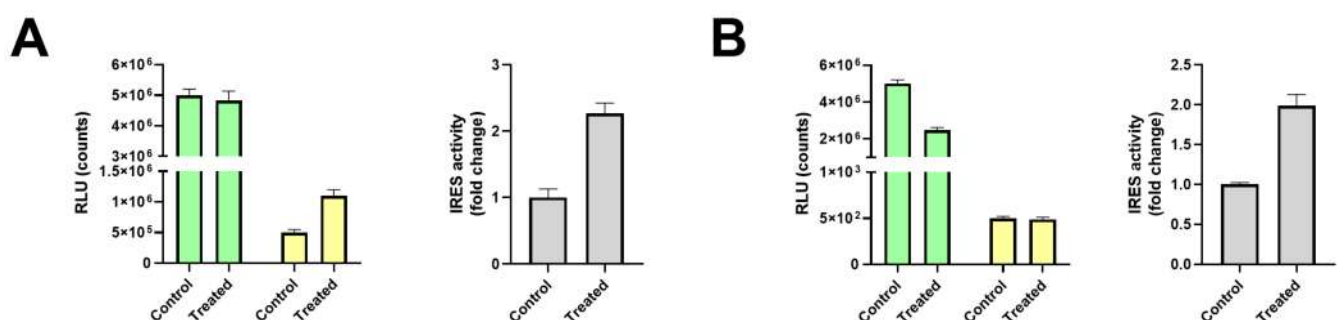


Figure 5. Potential misinterpretation of activity ratios without individual cistron data. Reporting of ratios alone can be misleading due to different underlying reasons for regulation. For example: (A) IRES ratio induction, caused by an increase of second cistron activity. (B) IRES ratio induction, due to a decrease of first cistron activity. Note that IRES activity in this hypothetical example is very low and might be close to the detection limit or background levels. Naturally, both first and second cistrons can also be induced or reduced, wherein slight differences can lead to IRES induction or repression. Additional normalization strategies can be used to correct for changes in cap translation (monocistronic β Gal co-transfection), cell number, or protein content. Graphs were generated for illustration purposes using hypothetical data, on the basis of our own experience with the bicistronic reporter assay.

Normalization of bicistronic reporter data is not always performed, and this depends largely on the study purpose. For bicistronic reporter experiments where the activity of the element between the two cistrons is not under investigation, such normalization is often not used. In general, care should be taken to prevent misinterpretation related to transcriptional activity, cell viability, cryptic splicing, or cryptic promoter activity. The fact that such limitations have mostly been described within the IRES field using Fluc/Rluc reporters does not imply that these issues do not take place in other fields or other reporter configurations. For most cellular studies, it is feasible to normalize to cell number (DNA content), total protein content, or cell viability, and such normalization approaches would certainly strengthen data interpretation.

A control condition (vehicle or non-treated) is commonly used in studies on IRES translation regulation following a stimulus or change in environment. Negative control plasmids are often used for investigating new 5' UTRs for potential CITE or IRES activity, and we consider this as an important control that could be more widely used. In addition, the use of a positive control, such as the EMCV IRES, would provide an elegant reference point. Additional hairpin structures 5' of the IRES, sometimes in combination with a monocistronic reporter, are often used to strengthen the cap-independent translation data (cf. Figure 2A). It is unfortunate that the most often used negative control hairpin sequences (Δ EMCV [239], P53 hairpin [240,241]) are challenging to trace down in the literature, although they might be well known in the field. Reporter mRNA levels or integrity are often verified in translational studies employing plasmid DNA, which provides additional indication for IRES or CITE activity. Northern blotting has not commonly been performed to address IRES reporter integrity since 2010, but (q)PCR-based techniques can be easily implemented.

In summary, on the basis of our literature review, we encourage researchers to adhere to the following recommendations for publication of bicistronic reporter data:

- (1) Make plasmid backbones and IRES sequences available upon publication.
- (2) Carefully describe transfection procedures and the timeline of the experiment.
- (3) Report both the ratio and individual cistron data.
- (4) Make raw data available, including background signals.
- (5) Verify whether treatments affect cell number, total protein content, or cell viability.
- (6) For cellular IRES translational studies, an empty control plasmid to define background signals; a promoterless control plasmid to assess cryptic promoter activity of the IRES; and verification of equal and intact mRNA levels by RT-qPCR and RT-PCR, respectively, are feasible and represent important controls.

More stringent controls have been recommended for the identification of eukaryotic IRESs (reviewed in [6,14]). These recommendations center on favoring mRNA transfections over plasmid DNA transfections and the generation of an extensive set of control mRNAs. These include an empty control (or inverted 5' UTR), a proximal stable hairpin, and monocistronic variants with and without cap. In our literature search, we found only four articles that adhered to these recommendations [43,160,165,205]. In addition, it is known that eukaryotic IRESs or CITEs have strongly reduced activity in mRNA transfection experiments, which has been used to question their existence or to argue for the existence of a nuclear experience of the mRNA [14,242]. Bicistronic reporters were often combined with monocistronic variants and an empty control plasmid, but introduction of a hairpin and cap vs. uncapped comparisons were rare. Naturally, once novel CITE or IRESs are established, follow-up studies might not require the same set of controls. However, our recommendations 1–6 would still be applicable for slightly different IRES sequences and different plasmid backbones. Finally, this study also has broader relevance for the optimization of bicistronic reporter use to drive transgene expression.

4. Methods

Search Strategy and Inclusion Criteria

We were interested in reported methodological details, data representation, normalization, and control experiments in articles on bicistronic reporters. Since IRESs are historically the most utilized and studied bicistronic elements, we focused on this in particular. Where possible we applied the PRISMA guidelines for a systematic review [243].

Pubmed, a repository of over 30 million citations to MEDLINE; life science journals; and online books were systematically searched with the keywords “IRES + bicistronic + reporter” and “IRES + dicistronic + reporter”. The number of identified articles was 257, and these were published between 1994 and 2020 (Table S1). The keywords “bicistronic + reporter” and “dicistronic + reporter” led to a large number of articles (571 studies, published between 1989 and 2020). It should be noted that we covered a significant portion of these articles (257/571) by including the IRES search term, while keeping the number of articles manageable. Although the term CITE has recently gained popularity, replacing IRES with CITE in the search terms yielded one article.

Review articles were excluded from analysis, since they lack new data and methodological procedures (Figure 1, screening). In addition, we focused on adherent eukaryotic cell systems, and therefore bacterial, fungal, and yeast studies were excluded (Figure 1, eligibility). Substantial lack of methodological details (such as transfection method and plasmid information) was also a reason for exclusion of the article for further analysis. Finally, the article should contain a bicistronic reporter construct with an IRES or 5' UTR. Therefore, studies were excluded if they utilized an IRES merely to drive an antibiotic resistance gene for selection and not for reporter purposes. Due to strong methodological differences between in vivo and in vitro translation assays, we dedicated a separate paragraph to the cell-free translation system. Three independent researchers (G.G.H.v.d.A., F.Z., and L.v.d.V.) excluded studies on the basis of the inclusion criteria for cellular systems, while two other independent researchers checked this for the cell-free translation system (F.Z., L.M.).

The extracted parameters that we defined and to which assay (in vitro or in vivo translation) they apply are indicated in Table 1. An arbitrary selection was made for visualization in pie charts. Certain parameters (e.g., co-factors in in vitro translation) were so little reported that we refrained from reviewing them here.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/ijms22105193/s1>, Table S1: Overview of identified and included research articles.

Author Contributions: Conceptualization, G.G.H.v.d.A., F.Z., L.M., and T.J.M.W.; methodology, G.G.H.v.d.A. and F.Z.; formal analysis, G.G.H.v.d.A., F.Z., B.A.C.H., L.v.d.V., and M.M.J.C.; data curation, G.G.H.v.d.A., F.Z., and L.v.d.V.; writing—original draft preparation, G.G.H.v.d.A. and F.Z.; writing—review and editing, G.G.H.v.d.A., F.Z., B.A.C.H., M.M.J.C., L.M., and T.J.M.W.; visualization, G.G.H.v.d.A. and F.Z.; funding acquisition, F.Z., L.M., and T.J.M.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Dutch Arthritis Foundation (grant LLP14), Stichting de Weijerhorst (Bewegen zonder Pijn), TTW/NWO Perspectief (P15–23; William Hunter revisited), Fondazione AIRC per la Ricerca sul Cancro (AIRC), IG 21562 to L.M. with a fellowship to F.Z.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article or supplementary material.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Schenborn, E.; Groskretz, D. Reporter Gene Vectors and Assays. *Mol. Biotechnol.* **1999**, *13*, 29–44. [[CrossRef](#)]
2. Robison, L.R.; Seligsohn, R.; Lerner, S.A. Simplified Radioenzymatic Assay for Chloramphenicol. *Antimicrob. Agents Chemother.* **1978**, *13*, 25–29. [[CrossRef](#)] [[PubMed](#)]
3. Ow, D.W.; de Wet, J.R.; Helinski, D.R.; Howell, S.H.; Wood, K.V.; Deluca, M. Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* **1986**, *234*, 856–859. [[CrossRef](#)] [[PubMed](#)]
4. Inouye, S.; Tsuji, F.I. Aequorea green fluorescent protein. Expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS Lett.* **1994**, *341*, 277–280. [[CrossRef](#)]
5. Jing, Y.; Simmer, P.; Feng, J.Q. Cell Lineage Tracing: Colocalization of Cell Lineage Markers with a Fluorescent Reporter. *Methods Mol. Biol.* **2021**, *2230*, 325–335.
6. Terenin, I.M.; Smirnova, V.V.; Andreev, D.E.; Dmitriev, S.E.; Shatsky, I.N. A researcher's guide to the galaxy of IRESs. *Cell. Mol. Life Sci. CMLS.* **2017**, *74*, 1431–1455. [[CrossRef](#)]
7. Blay, V.; Tolani, B.; Ho, S.P.; Arkin, M.R. High-Throughput Screening: Today's biochemical and cell-based approaches. *Drug Discov. Today* **2020**, *25*, 1807–1821. [[CrossRef](#)]
8. Kim, J.H.; Lee, S.R.; Li, L.H.; Park, H.J.; Park, J.H.; Lee, K.Y.; Choi, S.Y. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. *PLoS ONE* **2011**, *6*, e18556. [[CrossRef](#)]
9. Jang, S.K.; Davies, M.V.; Kaufman, R.J.; Wimmer, E. Initiation of protein synthesis by internal entry of ribosomes into the 5' non-translated region of encephalomyocarditis virus RNA in vivo. *J. Virol.* **1989**, *63*, 1651–1660. [[CrossRef](#)]
10. Jang, S.K.; Kräusslich, H.G.; Nicklin, M.J.; Duke, G.M.; Palmenberg, A.C.; Wimmer, E. A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J. Virol.* **1988**, *62*, 2636–2643. [[CrossRef](#)]
11. Kwan, T.; Thompson, S.R. Noncanonical Translation Initiation in Eukaryotes. In *Cold Spring Harbor Perspectives in Biology*; Cold Spring Harbor Laboratory Press: New York, NY, USA, 2019; Volume 11.
12. Mokrejš, M.; Mašek, T.; Vopálenský, V.; Hlubuček, P.; Delbos, P.; Pospíšek, M. IRESite—A tool for the examination of viral and cellular internal ribosome entry sites. *Nucleic Acids Res.* **2009**, *38*, D131–D136. [[CrossRef](#)]
13. Godet, A.-C.; David, F.; Hantelys, F.; Tatin, F.; Lacazette, E.; Garmy-Susini, B.; Prats, A.-C. IRES Trans-Acting Factors, Key Actors of the Stress Response. *Int. J. Mol. Sci.* **2019**, *20*, 924. [[CrossRef](#)]
14. Jackson, R.J. The Current Status of Vertebrate Cellular mRNA IRESs. *Cold Spring Harb. Perspect. Biol.* **2013**, *5*, a011569. [[CrossRef](#)]
15. Van Eden, M.E.; Byrd, M.P.; Sherrill, K.W.; Lloyd, R.E. Demonstrating internal ribosome entry sites in eukaryotic mRNAs using stringent RNA test procedures. *RNA* **2004**, *10*, 720–730. [[CrossRef](#)]
16. Yang, Y.; Wang, Z. IRES-mediated cap-independent translation, a path leading to hidden proteome. *J. Mol. Cell Biol.* **2019**, *11*, 911–919. [[CrossRef](#)]
17. Loughran, G.; Howard, M.T.; Firth, A.E.; Atkins, J.F. Avoidance of reporter assay distortions from fused dual reporters. *RNA* **2017**, *23*, 1285–1289. [[CrossRef](#)]
18. Penzo, M.; Carnicelli, D.; Montanaro, L.; Brigotti, M. A reconstituted cell-free assay for the evaluation of the intrinsic activity of purified human ribosomes. *Nat. Protoc.* **2016**, *11*, 1309–1325. [[CrossRef](#)]
19. Atkins, J.F.; Loughran, G.; Bhatt, P.R.; Firth, A.E.; Baranov, P.V. Ribosomal frameshifting and transcriptional slippage: From genetic steganography and cryptography to adventitious use. *Nucleic Acids Res.* **2016**, *44*, 7007–7078. [[CrossRef](#)]
20. Jacobs, J.L.; Dinman, J.D. Systematic analysis of bicistronic reporter assay data. *Nucleic Acids Res.* **2004**, *32*, e160. [[CrossRef](#)]
21. Ray, P.S.; Grover, R.; Das, S. Two internal ribosome entry sites mediate the translation of p53 isoforms. *EMBO Rep.* **2006**, *7*, 404–410. [[CrossRef](#)]
22. Weingarten-Gabbay, S.; Segal, E. Toward a systematic understanding of translational regulatory elements in human and viruses. *RNA Biol.* **2016**, *13*, 927–933. [[CrossRef](#)] [[PubMed](#)]
23. Weingarten-Gabbay, S.; Elias-Kirma, S.; Nir, R.; Gritsenko, A.A.; Stern-Ginossar, N.; Yakhini, Z.; Segal, E. Comparative genetics. Systematic discovery of cap-independent translation sequences in human and viral genomes. *Science* **2016**, *351*, aad4939. [[CrossRef](#)] [[PubMed](#)]
24. Weingarten-Gabbay, S.; Nir, R.; Lubliner, S.; Sharon, E.; Kalma, Y.; Weinberger, A.; Segal, E. Systematic interrogation of human promoters. *Genome Res.* **2019**, *29*, 171–183. [[CrossRef](#)] [[PubMed](#)]
25. Calamita, P.; Gatti, G.; Miluzio, A.; Scagliola, A.; Biffo, S. Translating the Game: Ribosomes as Active Players. *Front. Genet.* **2018**, *9*, 533. [[CrossRef](#)]
26. Horos, R.; Ijspeert, H.; Pospisilova, D.; Sendtner, R.; Andrieu-Soler, C.; Taskesen, E.; von Lindern, M. Ribosomal deficiencies in Diamond-Blackfan anemia impair translation of transcripts essential for differentiation of murine and human erythroblasts. *Blood* **2012**, *119*, 262–272. [[CrossRef](#)]
27. Penzo, M.; Rocchi, L.; Brugiare, S.; Carnicelli, D.; Onofrillo, C.; Couté, Y.; Brigotti, M.; Montanaro, L. Human ribosomes from cells with reduced dyskerin levels are intrinsically altered in translation. *FASEB J.* **2015**, *29*, 3472–3482. [[CrossRef](#)]
28. Marchand, V.; Pichot, F.; Neybecker, P.; Ayadi, L.; Bourguignon-Igel, V.; Wacheul, L.; Lafontaine, D.L.J.; Pinzano, A.; Helm, M.; Motorin, Y. HydraPsiSeq: A method for systematic and quantitative mapping of pseudouridines in RNA. *Nucleic Acids Res.* **2020**, *48*, e110. [[CrossRef](#)]

29. Galvanin, A.; Ayadi, L.; Helm, M.; Motorin, Y.; Marchand, V. Mapping and Quantification of tRNA 2'-O-Methylation by Ribo-MethSeq. *Methods Mol. Biol.* **2019**, *1870*, 273–295.
30. Wiener, D.; Schwartz, S. The epitranscriptome beyond m(6)A. In *Nature Reviews Genetics*; Nature Research: London, UK, 2020.
31. Jonkhout, N.; Tran, J.; Smith, M.A.; Schonrock, N.; Mattick, J.S.; Novoa, E.M. The RNA modification landscape in human disease. *RNA* **2017**, *23*, 1754–1769. [[CrossRef](#)]
32. Li, D.; Wang, J. Ribosome heterogeneity in stem cells and development. *J. Cell Biol.* **2020**, *219*, e202001108. [[CrossRef](#)]
33. Venturi, G.; Montanaro, L. How Altered Ribosome Production Can Cause or Contribute to Human Disease: The Spectrum of Ribosomopathies. *Cells* **2020**, *9*, 2300. [[CrossRef](#)]
34. Piskol, R.; Melo, F.D.S.E. Colon Cancer Heterogeneity: Welcome to the RiboZone. *Cell Stem Cell* **2020**, *26*, 797–799. [[CrossRef](#)]
35. Catez, F.; Venezia, N.D.; Marcel, V.; Zorbas, C.; Lafontaine, D.L.J.; Diaz, J.-J. Ribosome biogenesis: An emerging druggable pathway for cancer therapeutics. *Biochem. Pharmacol.* **2019**, *159*, 74–81. [[CrossRef](#)]
36. Ford, D. Ribosomal heterogeneity—A new inroad for pharmacological innovation. *Biochem. Pharmacol.* **2020**, *175*, 113874. [[CrossRef](#)]
37. Whetter, L.E.; Day, S.P.; Elroy-Stein, O.; Brown, E.A.; Lemon, S.M. Low efficiency of the 5' nontranslated region of hepatitis A virus RNA in directing cap-independent translation in permissive monkey kidney cells. *J. Virol.* **1994**, *68*, 5253–5263. [[CrossRef](#)]
38. Schultz, D.E.; Honda, M.; Whetter, L.E.; McKnight, K.L.; Lemon, S.M. Mutations within the 5' nontranslated RNA of cell culture-adapted hepatitis A virus which enhance cap-independent translation in cultured African green monkey kidney cells. *J. Virol.* **1996**, *70*, 1041–1049. [[CrossRef](#)]
39. Buratti, E.; Gerotto, M.; Pontisso, P.; Alberti, A.; Tisminetzky, S.; Baralle, F. In vivo translational efficiency of different hepatitis C virus 5'-UTRs. *FEBS Lett.* **1997**, *411*, 275–280. [[CrossRef](#)]
40. Chon, S.K.; Perez, D.R.; Donis, R.O. Genetic Analysis of the Internal Ribosome Entry Segment of Bovine Viral Diarrhea Virus. *Virology* **1998**, *251*, 370–382. [[CrossRef](#)]
41. Collier, A.J.; Tang, S.; Elliott, R.M. Translation efficiencies of the 5' untranslated region from representatives of the six major genotypes of hepatitis C virus using a novel bicistronic reporter assay system. *J. Gen. Virol.* **1998**, *79*, 2359–2366. [[CrossRef](#)]
42. Gan, W.; La Celle, M.; Rhoads, R.E. Functional Characterization of the Internal Ribosome Entry Site of eIF4G mRNA. *J. Biol. Chem.* **1998**, *273*, 5006–5012. [[CrossRef](#)]
43. Huez, I.; Créancier, L.; Audigier, S.; Gensac, M.-C.; Prats, A.-C.; Prats, H. Two Independent Internal Ribosome Entry Sites Are Involved in Translation Initiation of Vascular Endothelial Growth Factor mRNA. *Mol. Cell. Biol.* **1998**, *18*, 6178–6190. [[CrossRef](#)]
44. Hwang, L.-H.; Hsieh, C.-L.; Yen, A.; Chung, Y.-L.; Chen, D.S. Involvement of the 5' Proximal Coding Sequences of Hepatitis C Virus with Internal Initiation of Viral Translation. *Biochem. Biophys. Res. Commun.* **1998**, *252*, 455–460. [[CrossRef](#)]
45. Lopez de Quinto, S.; Martinez-Salas, E. Parameters influencing translational efficiency in aphthovirus IRES-based bicistronic expression vectors. *Gene* **1998**, *217*, 51–56. [[CrossRef](#)]
46. Miller, D.L.; Dibbens, J.A.; Damert, A.; Risau, W.; Vadas, M.A.; Goodall, G.J. The vascular endothelial growth factor mRNA contains an internal ribosome entry site. *FEBS Lett.* **1998**, *434*, 417–420. [[CrossRef](#)]
47. Roberts, L.O.; Seamons, R.A.; Belsham, G.J. Recognition of picornavirus internal ribosome entry sites within cells; influence of cellular and viral proteins. *RNA* **1998**, *4*, 520–529. [[CrossRef](#)]
48. Stein, I.; Itin, A.; Einat, P.; Skaliter, R.; Grossman, Z.; Keshet, E. Translation of Vascular Endothelial Growth Factor mRNA by Internal Ribosome Entry: Implications for Translation under Hypoxia. *Mol. Cell. Biol.* **1998**, *18*, 3112–3119. [[CrossRef](#)]
49. Stoneley, M.; Paulin, F.E.; Le Quesne, J.P.; Chappell, S.A.; Willis, A.E. C-Myc 5' untranslated region contains an internal ribosome entry segment. *Oncogene* **1998**, *16*, 423–428. [[CrossRef](#)]
50. Attal, J.; Théron, M.-C.; Houdebine, L.M. The optimal use of IRES (internal ribosome entry site) in expression vectors. *Genet. Anal. Biomol. Eng.* **1999**, *15*, 161–165. [[CrossRef](#)]
51. De Gregorio, E.; Preiss, T.; Hentze, M.W. Translation driven by an eIF4G core domain in vivo. *EMBO J.* **1999**, *18*, 4865–4874. [[CrossRef](#)] [[PubMed](#)]
52. Finkelstein, Y.; Faktor, O.; Elroy-Stein, O.; Levi, B.-Z. The use of bi-cistronic transfer vectors for the baculovirus expression system. *J. Biotechnol.* **1999**, *75*, 33–44. [[CrossRef](#)]
53. Grace, K.; Gartland, M.; Karayiannis, P.; McGarvey, M.J.; Clarke, B. The 5' untranslated region of GB virus B shows functional similarity to the internal ribosome entry site of hepatitis C virus. *J. Gen. Virol.* **1999**, *80*, 2337–2341. [[CrossRef](#)] [[PubMed](#)]
54. Schiavi, A.; Hudder, A.; Werner, R. Connexin43 mRNA contains a functional internal ribosome entry site. *FEBS Lett.* **1999**, *464*, 118–122. [[CrossRef](#)]
55. Tang, S.; Collier, A.J.; Elliott, R.M. Alterations to both the primary and predicted secondary structure of stem-loop IIIc of the hepatitis C virus 1b 5' untranslated region (5'UTR) lead to mutants severely defective in translation which cannot be complemented in trans by the wild-type 5'UTR sequence. *J. Virol.* **1999**, *73*, 2359–2364.
56. Zhu, J.; Musco, M.L.; Grace, M.J. Three-color flow cytometry analysis of tricistronic expression of eBFP, eGFP, and eYFP using EMCV-IRES linkages. *Cytometry.* **1999**, *37*, 51–59. [[CrossRef](#)]
57. Chappell, S.A.; Edelman, G.M.; Mauro, V.P. A 9-nt segment of a cellular mRNA can function as an internal ribosome entry site (IRES) and when present in linked multiple copies greatly enhances IRES activity. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 1536–1541. [[CrossRef](#)]
58. Créancier, L.; Morello, D.; Mercier, P.; Prats, A.-C. Fibroblast Growth Factor 2 Internal Ribosome Entry Site (Ires) Activity Ex Vivo and in Transgenic Mice Reveals a Stringent Tissue-Specific Regulation. *J. Cell Biol.* **2000**, *150*, 275–281. [[CrossRef](#)]

59. Henis-Korenblit, S.; Strumpf, N.L.; Goldstaub, D.; Kimchi, A. A Novel Form of DAP5 Protein Accumulates in Apoptotic Cells as a Result of Caspase Cleavage and Internal Ribosome Entry Site-Mediated Translation. *Mol. Cell. Biol.* **2000**, *20*, 496–506. [[CrossRef](#)]
60. Hinton, T.M.; Li, F.; Crabb, B.S. Internal Ribosomal Entry Site-Mediated Translation Initiation in Equine Rhinitis A Virus: Similarities to and Differences from That of Foot-and-Mouth Disease Virus. *J. Virol.* **2000**, *74*, 11708–11716. [[CrossRef](#)]
61. Honda, M.; Kaneko, S.; Matsushita, E.; Kobayashi, K.; Abell, G.A.; Lemon, S.M. Cell cycle regulation of hepatitis C virus internal ribosomal entry site-directed translation. *Gastroenterology* **2000**, *118*, 152–162. [[CrossRef](#)]
62. Hudder, A.; Werner, R. Analysis of a Charcot-Marie-Tooth Disease Mutation Reveals an Essential Internal Ribosome Entry Site Element in the Connexin-32 Gene. *J. Biol. Chem.* **2000**, *275*, 34586–34591. [[CrossRef](#)]
63. Lerat, H.; Shimizu, Y.K.; Lemon, S.M. Cell Type-Specific Enhancement of Hepatitis C Virus Internal Ribosome Entry Site-Directed Translation due to 5' Nontranslated Region Substitutions Selected during Passage of Virus in Lymphoblastoid Cells. *J. Virol.* **2000**, *74*, 7024–7031. [[CrossRef](#)]
64. Oumard, A.; Hennecke, M.; Hauser, H.; Nourbakhsh, M. Translation of NRF mRNA Is Mediated by Highly Efficient Internal Ribosome Entry. *Mol. Cell. Biol.* **2000**, *20*, 2755–2759. [[CrossRef](#)]
65. Pozner, A.; Goldenberg, D.; Negreanu, V.; Le, S.-Y.; Elroy-Stein, O.; Levanon, D.; Groner, Y. Transcription-Coupled Translation Control of AML1/RUNX1 Is Mediated by Cap- and Internal Ribosome Entry Site-Dependent Mechanisms. *Mol. Cell. Biol.* **2000**, *20*, 2297–2307. [[CrossRef](#)]
66. Rijnbrand, R.; Abell, G.; Lemon, S.M. Mutational Analysis of the GB Virus B Internal Ribosome Entry Site. *J. Virol.* **2000**, *74*, 773–783. [[CrossRef](#)]
67. Wang, T.-H.; Rijnbrand, R.C.A.; Lemon, S.M. Core Protein-Coding Sequence, but Not Core Protein, Modulates the Efficiency of Cap-Independent Translation Directed by the Internal Ribosome Entry Site of Hepatitis C Virus. *J. Virol.* **2000**, *74*, 11347–11358. [[CrossRef](#)]
68. Bieleski, L.; Talbot, S.J. Kaposi's sarcoma-associated herpesvirus vCyclin open reading frame contains an internal ribosome entry site. *J. Virol.* **2001**, *75*, 1864–1869. [[CrossRef](#)]
69. Chappell, S.A.; Owens, G.C.; Mauro, V.P. A 5' Leader of Rbm3, a Cold Stress-induced mRNA, Mediates Internal Initiation of Translation with Increased Efficiency under Conditions of Mild Hypothermia. *J. Biol. Chem.* **2001**, *276*, 36917–36922. [[CrossRef](#)]
70. Chiang, P.-W.; Carpenter, L.E.; Hagerman, P.J. The 5'-Untranslated Region of the FMR1 Message Facilitates Translation by Internal Ribosome Entry. *J. Biol. Chem.* **2001**, *276*, 37916–37921. [[CrossRef](#)]
71. Furler, S.; Paterna, J.-C.; Weibel, M.; Bueler, H. Recombinant AAV vectors containing the foot and mouth disease virus 2A sequence confer efficient bicistronic gene expression in cultured cells and rat substantia nigra neurons. *Gene Ther.* **2001**, *8*, 864–873. [[CrossRef](#)]
72. Grundhoff, A.; Ganem, D. Mechanisms governing expression of the v-FLIP gene of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* **2001**, *75*, 1857–1863. [[CrossRef](#)]
73. Hennecke, M.; Kwissa, M.; Metzger, K.; Oumard, A.; Kröger, A.; Schirmbeck, R.; Reimann, J.; Hauser, H. Composition and arrangement of genes define the strength of IRES-driven translation in bicistronic mRNAs. *Nucleic Acids Res.* **2001**, *29*, 3327–3334. [[CrossRef](#)] [[PubMed](#)]
74. Krüger, M.; Beger, C.; Welch, P.J.; Barber, J.R.; Manns, M.P.; Wong-Staal, F. Involvement of Proteasome α -Subunit PSMA7 in Hepatitis C Virus Internal Ribosome Entry Site-Mediated Translation. *Mol. Cell. Biol.* **2001**, *21*, 8357–8364. [[CrossRef](#)] [[PubMed](#)]
75. Pinkstaff, J.K.; Chappell, S.A.; Mauro, V.P.; Edelman, G.M.; Krushel, L.A. Internal initiation of translation of five dendritically localized neuronal mRNAs. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 2770–2775. [[CrossRef](#)] [[PubMed](#)]
76. Schwarz, E.J.; Reger, R.L.; Alexander, G.M.; Class, R.; Azizi, S.A.; Prockop, D.J. Rat marrow stromal cells rapidly transduced with a self-inactivating retrovirus synthesize L-DOPA in vitro. *Gene Ther.* **2001**, *8*, 1214–1223. [[CrossRef](#)]
77. Venkatesan, A.; Dasgupta, A. Novel Fluorescence-Based Screen to Identify Small Synthetic Internal Ribosome Entry Site Elements. *Mol. Cell. Biol.* **2001**, *21*, 2826–2837. [[CrossRef](#)]
78. Grobe, K.; Esko, J.D. Regulated translation of heparan sulfate N-acetylglucosamine N-deacetylase/n-sulfotransferase isozymes by structured 5'-untranslated regions and internal ribosome entry sites. *J. Biol. Chem.* **2002**, *277*, 30699–30706. [[CrossRef](#)]
79. Kato, J.; Kato, N.; Moriyama, M.; Goto, T.; Taniguchi, H.; Shiratori, Y.; Omata, M. Interferons Specifically Suppress the Translation from the Internal Ribosome Entry Site of Hepatitis C Virus through a Double-Stranded RNA-Activated Protein Kinase-Independent Pathway. *J. Infect. Dis.* **2002**, *186*, 155–163. [[CrossRef](#)]
80. Kato, J.; Kato, N.; Yoshida, H.; Ono-Nita, S.K.; Shiratori, Y.; Omata, M. Hepatitis C virus NS4A and NS4B proteins suppress translation in vivo. *J. Med. Virol.* **2001**, *66*, 187–199. [[CrossRef](#)]
81. Koev, G.; Duncan, R.F.; Lai, M.M. Hepatitis C virus IRES-dependent translation is insensitive to an eIF2 α -independent mechanism of inhibition by interferon in hepatocyte cell lines. *Virology* **2002**, *297*, 195–202. [[CrossRef](#)]
82. Lang, K.J.D.; Kappel, A.; Goodall, G.J. Hypoxia-inducible Factor-1 α mRNA Contains an Internal Ribosome Entry Site That Allows Efficient Translation during Normoxia and Hypoxia. *Mol. Biol. Cell* **2002**, *13*, 1792–1801. [[CrossRef](#)]
83. Morrish, B.C.; Rumsby, M.G. The 5' untranslated region of protein kinase Cdelta directs translation by an internal ribosome entry segment that is most active in densely growing cells and during apoptosis. *Mol. Cell. Biol.* **2002**, *22*, 6089–6099. [[CrossRef](#)]
84. Shimazaki, T.; Honda, M.; Kaneko, S.; Kobayashi, K. Inhibition of internal ribosomal entry site-directed translation of HCV by recombinant IFN- α correlates with a reduced La protein. *Hepatology* **2002**, *35*, 199–208. [[CrossRef](#)]

85. van der Velden, A.W.; van Nierop, K.; Voorma, H.O.; Thomas, A.A. Ribosomal scanning on the highly structured insulin-like growth factor II-leader. *Int. J. Biochem. Cell. Biol.* **2002**, *34*, 286–297. [[CrossRef](#)]
86. Zhang, J.; Yamada, O.; Yoshida, H.; Iwai, T.; Araki, H. Autogenous Translational Inhibition of Core Protein: Implication for Switch from Translation to RNA Replication in Hepatitis C Virus. *Virology* **2002**, *293*, 141–150. [[CrossRef](#)]
87. Coleman, H.M.; Brierley, I.; Stevenson, P.G. An Internal Ribosome Entry Site Directs Translation of the Murine Gammaherpesvirus 68 MK3 Open Reading Frame. *J. Virol.* **2003**, *77*, 13093–13105. [[CrossRef](#)]
88. De Pietri Tonelli, D.; Mihailovich, M.; Schnurbus, R.; Pesole, G.; Grohovaz, F.; Zacchetti, D. Translational control of Scamper expression via a cell-specific internal ribosome entry site. *Nucleic Acids Res.* **2003**, *31*, 2508–2513. [[CrossRef](#)]
89. Figueroa, C.; Vojtek, A.B. Akt negatively regulates translation of the ternary complex factor Elk-1. *Oncogene* **2003**, *22*, 5554–5561. [[CrossRef](#)]
90. He, Y.; Yan, W.; Coito, C.; Li, Y.; Gale, M.; Katze, M.G. The regulation of hepatitis C virus (HCV) internal ribosome-entry site-mediated translation by HCV replicons and nonstructural proteins. *J. Gen. Virol.* **2003**, *84*, 535–543. [[CrossRef](#)]
91. Imbert, I.; Dimitrova, M.; Kien, F.; Kieny, M.P.; Schuster, C. Hepatitis C virus IRES efficiency is unaffected by the genomic RNA 3'NTR even in the presence of viral structural or non-structural proteins. *J. Gen. Virol.* **2003**, *84*, 1549–1557. [[CrossRef](#)]
92. Isaksson, Åsa; Berggren, M.; Ricksten, A. Epstein-Barr virus U leader exon contains an internal ribosome entry site. *Oncogene* **2003**, *22*, 572–581. [[CrossRef](#)]
93. Kim, Y.K.; Lee, S.H.; Kim, C.S.; Seol, S.K.; Jang, S.K. Long-range RNA-RNA interaction between the 5' nontranslated region and the core-coding sequences of hepatitis C virus modulates the IRES-dependent translation. *RNA* **2003**, *9*, 599–606. [[CrossRef](#)] [[PubMed](#)]
94. Kobayashi, N.; Saeki, K.; Yuo, A. Granulocyte-macrophage colony-stimulating factor and interleukin-3 induce cell cycle progression through the synthesis of c-Myc protein by internal ribosome entry site-mediated translation via phosphatidylinositol 3-kinase pathway in human factor-dependent leukemic cells. *Blood* **2003**, *102*, 3186–3195. [[PubMed](#)]
95. Masoumi, A.; Hanzlik, T.N.; Christian, P.D. Functionality of the 5'- and intergenic IRES elements of cricket paralysis virus in a range of insect cell lines, and its relationship with viral activities. *Virus Res.* **2003**, *94*, 113–120. [[CrossRef](#)]
96. Rubtsova, M.P.; Sizova, D.V.; Dmitriev, S.E.; Ivanov, D.S.; Prassolov, V.S.; Shatsky, I.N. Distinctive Properties of the 5'-Untranslated Region of Human Hsp70 mRNA. *J. Biol. Chem.* **2003**, *278*, 22350–22356. [[CrossRef](#)]
97. Thompson, S.R.; Sarnow, P. Enterovirus 71 contains a type I IRES element that functions when eukaryotic initiation factor eIF4G is cleaved. *Virology* **2003**, *315*, 259–266. [[CrossRef](#)]
98. Venkatesan, A.; Sharma, R.; Dasgupta, A. Cell cycle regulation of hepatitis C and encephalomyocarditis virus internal ribosome entry site-mediated translation in human embryonic kidney 293 cells. *Virus Res.* **2003**, *94*, 85–95. [[CrossRef](#)]
99. Vyas, J.; Elia, A.; Clemens, M.J. Inhibition of the protein kinase PKR by the internal ribosome entry site of hepatitis C virus genomic RNA. *RNA* **2003**, *9*, 858–870. [[CrossRef](#)]
100. Xiao, L.; Liu, P.; Sobue, T.; Lichtler, A.; Coffin, J.D.; Hurley, M.M. Effect of overexpressing fibroblast growth factor 2 protein isoforms in osteoblastic ROS 17/2.8 cells. *J. Cell. Biochem.* **2003**, *89*, 1291–1301. [[CrossRef](#)]
101. Xiao, Z.-S.; Simpson, L.G.; Quarles, L.D. IRES-dependent translational control of Cbfa1/Runx2 expression. *J. Cell. Biochem.* **2003**, *88*, 493–505. [[CrossRef](#)]
102. Forton, D.M.; Karayiannis, P.; Mahmud, N.; Taylor-Robinson, S.D.; Thomas, H.C. Identification of unique hepatitis C virus quasi-species in the central nervous system and comparative analysis of internal translational efficiency of brain, liver, and serum variants. *J. Virol.* **2004**, *78*, 5170–5183. [[CrossRef](#)]
103. Teshima-Kondo, S.; Kondo, K.; Prado-Lourenco, L.; Gabriela Gonzalez-Herrera, I.; Rokutan, K.; Bayard, F.; Prats, A.C. Hyperglycemia upregulates translation of the fibroblast growth factor 2 mRNA in mouse aorta via internal ribosome entry site. *FASEB J.* **2004**, *18*, 1583–1585. [[CrossRef](#)] [[PubMed](#)]
104. Tischendorf, J.J.W.; Beger, C.; Korf, M.; Manns, M.P. Polypyrimidine tract-binding protein (PTB) inhibits Hepatitis C virus internal ribosome entry site (HCV IRES)-mediated translation, but does not affect HCV replication. *Arch. Virol.* **2003**, *149*, 1. [[CrossRef](#)] [[PubMed](#)]
105. Zhou, J.; Callapina, M.; Goodall, G.J.; Brune, B. Functional integrity of nuclear factor kappaB, phosphatidylinositol 3'-kinase, and mitogen-activated protein kinase signaling allows tumor necrosis factor alpha-evoked Bcl-2 expression to provoke internal ribosome entry site-dependent translation of hypoxia-inducible factor 1alpha. *Cancer Res.* **2004**, *64*, 9041–9048.
106. Bevitt, D.J.; Li, Z.; Lindrop, J.L.; Barker, M.D.; Clarke, M.P.; McKie, N. Analysis of full length ADAMTS6 transcript reveals alternative splicing and a role for the 5' untranslated region in translational control. *Gene* **2005**, *359*, 99–110. [[CrossRef](#)]
107. Cornelis, S.; Tinton, S.A.; Schepens, B.; Bruynooghe, Y.; Beyaert, R. UNR translation can be driven by an IRES element that is negatively regulated by polypyrimidine tract binding protein. *Nucleic Acids Res.* **2005**, *33*, 3095–3108. [[CrossRef](#)]
108. Czibener, C.; Alvarez, D.; Scodeller, E.; Gamarnik, A.V. Characterization of internal ribosomal entry sites of Triatoma virus. *J. Gen. Virol.* **2005**, *86*, 2275–2280. [[CrossRef](#)]
109. Holcik, M.; Graber, T.; Lewis, S.M.; Lefebvre, C.A.; Lacasse, E.; Baird, S. Spurious splicing within the XIAP 5' UTR occurs in the RLuc/Fluc but not the betagal/CAT bicistronic reporter system. *RNA* **2005**, *11*, 1605–1609. [[CrossRef](#)]
110. Kanda, T.; Yokosuka, O.; Imazeki, F.; Fujiwara, K.; Nagao, K.; Saisho, H. Amantadine inhibits hepatitis A virus internal ribosomal entry site-mediated translation in human hepatoma cells. *Biochem. Biophys. Res. Commun.* **2005**, *331*, 621–629. [[CrossRef](#)]

111. Kumimoto, H.; Yamane, Y.; Ishizaki, K. Two haplotypes of 5' untranslated region in L-myc gene with different internal ribosome entry site activity. *Int. J. Oncol.* **2005**, *26*, 287–293. [[CrossRef](#)]
112. Miura, P.; Thompson, J.; Chakkalakal, J.V.; Holcik, M.; Jasmin, B.J. The Utraphin A 5'-Untranslated Region Confers Internal Ribosome Entry Site-mediated Translational Control during Regeneration of Skeletal Muscle Fibers. *J. Biol. Chem.* **2005**, *280*, 32997–33005. [[CrossRef](#)]
113. Zhou, W.; Edelman, G.M.; Mauro, V.P. A positive feedback vector for identification of nucleotide sequences that enhance translation. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 6273–6278. [[CrossRef](#)]
114. Bert, A.G.; Grépin, R.; Vadas, M.A.; Goodall, G.J. Assessing IRES activity in the HIF-1 and other cellular 5' UTRs. *RNA* **2006**, *12*, 1074–1083. [[CrossRef](#)]
115. Bochkov, Y.A.; Palmenberg, A.C. Translational efficiency of EMCV IRES in bicistronic vectors is dependent upon IRES sequence and gene location. *Biotechniques* **2006**, *41*, 283–292. [[CrossRef](#)]
116. Elango, N.; Li, Y.; Shivshankar, P.; Katz, M.S. Expression of RUNX2 isoforms: Involvement of cap-dependent and cap-independent mechanisms of translation. *J. Cell. Biochem.* **2006**, *99*, 1108–1121. [[CrossRef](#)]
117. Lu, J.; Zhang, J.; Wang, X.; Jiang, H.; Liu, C.; Hu, Y. In vitro and in vivo identification of structural and sequence elements in the 5' untranslated region of Ectropis obliqua picorna-like virus required for internal initiation. *J. Gen. Virol.* **2006**, *87*, 3667–3677. [[CrossRef](#)]
118. MacCallum, P.R.; Jack, S.C.; Egan, P.A.; McDermott, B.T.; Elliott, R.M.; Chan, S.W. Cap-dependent and hepatitis C virus internal ribosome entry site-mediated translation are modulated by phosphorylation of eIF2 α under oxidative stress. *J. Gen. Virol.* **2006**, *87*, 3251–3262. [[CrossRef](#)]
119. Nie, M.; Htun, H. Different modes and potencies of translational repression by sequence-specific RNA–protein interaction at the 5'-UTR. *Nucleic Acids Res.* **2006**, *34*, 5528–5540. [[CrossRef](#)]
120. Yu, Y.; Alwine, J.C. 19S Late mRNAs of Simian Virus 40 Have an Internal Ribosome Entry Site Upstream of the Virion Structural Protein 3 Coding Sequence. *J. Virol.* **2006**, *80*, 6553–6558. [[CrossRef](#)] [[PubMed](#)]
121. Albagli-Curiel, O.; Lecluse, Y.; Pognonec, P.; Boulukos, K.E.; Martin, P. A new generation of pPRIG-based retroviral vectors. *BMC Biotechnol.* **2007**, *7*, 85. [[CrossRef](#)] [[PubMed](#)]
122. Chang, Q.; Bhatia, D.; Zhang, Y.; Meighan, T.; Castranova, V.; Shi, X.; Chen, F. Incorporation of an Internal Ribosome Entry Site-Dependent Mechanism in Arsenic-Induced GADD45 α Expression. *Cancer Res.* **2007**, *67*, 6146–6154. [[CrossRef](#)] [[PubMed](#)]
123. Jiang, H.; Coleman, J.; Miskimins, R.; Srinivasan, R.; Miskimins, W.K. Cap-independent translation through the p27 5'-UTR. *Nucleic Acids Res.* **2007**, *35*, 4767–4778. [[CrossRef](#)]
124. Jünemann, C.; Song, Y.; Bassili, G.; Goergen, D.; Henke, J.; Niepmann, M. Picornavirus Internal Ribosome Entry Site Elements Can Stimulate Translation of Upstream Genes. *J. Biol. Chem.* **2007**, *282*, 132–141. [[CrossRef](#)]
125. Kamrud, K.I.; Custer, M.; Dudek, J.M.; Owens, G.; Alterson, K.D.; Lee, J.S.; Groebner, J.L.; Smith, J.F. Alphavirus replicon approach to promoterless analysis of IRES elements. *Virology* **2007**, *360*, 376–387. [[CrossRef](#)]
126. Li, J.; Menzel, C.; Meier, D.; Zhang, C.; Dübel, S.; Jostock, T. A comparative study of different vector designs for the mammalian expression of recombinant IgG antibodies. *J. Immunol. Methods* **2007**, *318*, 113–124. [[CrossRef](#)]
127. Li, J.; Zhang, C.; Jostock, T.; Dübel, S. Analysis of IgG heavy chain to light chain ratio with mutant Encephalomyocarditis virus internal ribosome entry site. *Protein Eng. Des. Sel.* **2007**, *20*, 491–496. [[CrossRef](#)]
128. Orlinger, K.K.; Kofler, R.M.; Heinz, F.X.; Hoenninger, V.M.; Mandl, C.W. Selection and analysis of mutations in an encephalomyocarditis virus internal ribosome entry site that improve the efficiency of a bicistronic flavivirus construct. *J. Virol.* **2007**, *81*, 12619–12629. [[CrossRef](#)]
129. Petz, M.; Kozina, D.; Huber, H.; Siwiec, T.; Seipelt, J.; Sommergruber, W.; Mikulits, W. The leader region of Laminin B1 mRNA confers cap-independent translation. *Nucleic Acids Res.* **2007**, *35*, 2473–2482. [[CrossRef](#)]
130. Reboll, M.R.; Oumard, A.; Gazdag, A.C.; Renger, I.; Ritter, B.; Schwarzer, M.; Hauser, H.; Wood, M.; Yamada, M.; Resch, K.; et al. NRF IRES activity is mediated by RNA binding protein JKTBP1 and a 14-nt RNA element. *RNA* **2007**, *13*, 1328–1340. [[CrossRef](#)]
131. Sharma, A.; Masri, J.; Jo, O.D.; Bernath, A.; Martin, J.; Funk, A.; Gera, J. Protein Kinase C Regulates Internal Initiation of Translation of the GATA-4 mRNA following Vasopressin-induced Hypertrophy of Cardiac Myocytes. *J. Biol. Chem.* **2007**, *282*, 9505–9516. [[CrossRef](#)]
132. Siddappa, N.B.; Kashi, V.P.; Venkatramanan, M.; Balasiddaiah, A.; Jayasuryan, N.; Mahadevan, A.; Desai, A.; Satish, K.S.; Shankar, S.K.; Ravi, V.; et al. Gene Expression Analysis from Human Immunodeficiency Virus Type 1 Subtype C Promoter and Construction of Bicistronic Reporter Vectors. *AIDS Res. Hum. Retrovir.* **2007**, *23*, 1268–1278. [[CrossRef](#)]
133. Baranick, B.T.; Lemp, N.A.; Nagashima, J.; Hiraoka, K.; Kasahara, N.; Logg, C.R. Splicing mediates the activity of four putative cellular internal ribosome entry sites. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 4733–4738. [[CrossRef](#)]
134. Chen, Y.-J.; Zeng, S.-J.; Hsu, J.T.; Horng, J.-T.; Yang, H.-M.; Shih, S.-R.; Chu, Y.-T.; Wu, T.-Y. Amantadine as a regulator of internal ribosome entry site. *Acta Pharmacol. Sin.* **2008**, *29*, 1327–1333. [[CrossRef](#)] [[PubMed](#)]
135. D'Aiuto, L.; Robison, C.S.; Gigante, M.; Nwanegbo, E.; Shaffer, B.; Sukhwani, M.; Castro, C.A.; Chaillet, J.R. Human IL-12 p40 as a reporter gene for high-throughput screening of engineered mouse embryonic stem cells. *BMC Biotechnol.* **2008**, *8*, 52. [[CrossRef](#)] [[PubMed](#)]
136. Jean, D.; Rousselet, N.; Frade, R. Cathepsin L expression is up-regulated by hypoxia in human melanoma cells: Role of its 5'-untranslated region. *Biochem. J.* **2008**, *413*, 125–134. [[CrossRef](#)] [[PubMed](#)]

137. Kazadi, K.; Loeuillet, C.; Deutsch, S.; Ciuffi, A.; Muñoz, M.; Beckmann, J.S.; Moradpour, D.; Antonarakis, S.E.; Telenti, A. Genomic determinants of the efficiency of internal ribosomal entry sites of viral and cellular origin. *Nucleic Acids Res.* **2008**, *36*, 6918–6925. [[CrossRef](#)]
138. Meng, Z.; Jackson, N.L.; Choi, H.; King, P.H.; Emanuel, P.D.; Blume, S.W. Alterations in RNA-binding activities of IRES-regulatory proteins as a mechanism for physiological variability and pathological dysregulation of IGF-IR translational control in human breast tumor cells. *J. Cell. Physiol.* **2008**, *217*, 172–183. [[CrossRef](#)]
139. Miura, P.; Andrews, M.; Holcik, M.; Jasmin, B.J. IRES-Mediated Translation of Utrophin A Is Enhanced by Glucocorticoid Treatment in Skeletal Muscle Cells. *PLoS ONE* **2008**, *3*, e2309. [[CrossRef](#)]
140. Ottobriani, L.; Ciana, P.; Moresco, R.; Lecchi, M.; Belloli, S.; Martelli, C.; Todde, S.; Fazio, F.; Gambhir, S.S.; Maggi, A.; et al. Development of a bicistronic vector for multimodality imaging of estrogen receptor activity in a breast cancer model: Preliminary application. *Eur. J. Nucl. Med. Mol. Imaging* **2008**, *35*, 365–378. [[CrossRef](#)]
141. Sasaki, Y.; Sone, T.; Yahata, K.; Kishine, H.; Hotta, J.; Chesnut, J.D.; Honda, T.; Imamoto, F. WITHDRAWN: Multi-gene gateway clone design for expression of multiple heterologous genes in living cells: Eukaryotic clones containing two and three ORF multi-gene cassettes expressed from a single promoter. *J. Biotechnol.* **2005**, *136*, 103–112. [[CrossRef](#)]
142. Vazquez-Padron, R.I.; Pham, S.M.; Mateu, D.; Khan, S.; Aitouche, A. An internal ribosome entry site mediates the initiation of soluble guanylyl cyclase beta2 mRNA translation. *FEBS J.* **2008**, *275*, 3598–3607. [[CrossRef](#)]
143. Blaustein, M.; Quadrona, L.; Risso, G.; De La Mata, M.; Pelisch, F.; Srebrow, A. SF2/ASF regulates proteomic diversity by affecting the balance between translation initiation mechanisms. *J. Cell. Biochem.* **2009**, *107*, 826–833. [[CrossRef](#)]
144. Chan, S.-W.; Egan, P.A. Effects of hepatitis C virus envelope glycoprotein unfolded protein response activation on translation and transcription. *Arch. Virol.* **2009**, *154*, 1631–1640. [[CrossRef](#)]
145. Charnay, N.; Ivanyi-Nagy, R.; Soto-Rifo, R.; Ohlmann, T.; López-Lastra, M.; Darlix, J.-L. Mechanism of HIV-1 Tat RNA translation and its activation by the Tat protein. *Retrovirology* **2009**, *6*, 1–18. [[CrossRef](#)]
146. Chen, G.L.; Miller, G.M. 5'-Untranslated region of the tryptophan hydroxylase-2 gene harbors an asymmetric bidirectional pro-moter but not internal ribosome entry site in vitro. *Gene* **2009**, *435*, 53–62. [[CrossRef](#)]
147. Ibrahim, A.; Velde, G.V.; Reumers, V.; Toelen, J.; Thiry, I.; Vandeputte, C.; Vets, S.; Deroose, C.; Bormans, G.; Baekelandt, V.; et al. Highly Efficient Multicistronic Lentiviral Vectors with Peptide 2A Sequences. *Hum. Gene Ther.* **2009**, *20*, 845–860. [[CrossRef](#)]
148. Kamoshita, N.; Nomoto, A.; Rajbhandary, U.L. Translation Initiation from the Ribosomal A Site or the P Site, Dependent on the Conformation of RNA Pseudoknot I in Dicistrovirus RNAs. *Mol. Cell* **2009**, *35*, 181–190. [[CrossRef](#)]
149. Lourenco, S.; Boni, S.; Furling, D.; Cosset, F.-L.; Cahour, A. A cell-based bicistronic lentiviral reporter system for identification of inhibitors of the hepatitis C virus internal ribosome entry site. *J. Virol. Methods* **2009**, *158*, 152–159. [[CrossRef](#)]
150. Marschalek, A.; Finke, S.; Schwemmle, M.; Mayer, D.; Heimrich, B.; Stitz, L.; Conzelmann, K.K. Attenuation of rabies virus replication and virulence by picornavirus internal ribosome entry site elements. *J. Virol.* **2009**, *83*, 1911–1919. [[CrossRef](#)]
151. Saffran, H.A.; Smiley, J.R. The XIAP IRES activates 3' cistron expression by inducing production of monocistronic mRNA in the betagal/CAT bicistronic reporter system. *RNA* **2009**, *15*, 1980–1985. [[CrossRef](#)]
152. Spriggs, K.A.; Cobbold, L.C.; Ridley, S.H.; Coldwell, M.; Bottley, A.; Bushell, M.; Willis, A.E.; Siddle, K. The human insulin receptor mRNA contains a functional internal ribosome entry segment. *Nucleic Acids Res.* **2009**, *37*, 5881–5893. [[CrossRef](#)]
153. Tahiri-Alaoui, A.; Matsuda, D.; Xu, H.; Panagiotis, P.; Burman, L.; Lambeth, L.S.; Petherbridge, L.; James, W.; Mauro, V.; Nair, V. The 5' leader of the mRNA encoding the marek's disease virus serotype 1 pp14 protein contains an intronic internal ribosome entry site with allosteric properties. *J. Virol.* **2009**, *83*, 12769–12778. [[CrossRef](#)]
154. Wang, G.; Guo, X.; Silveyra, P.; Kimball, S.R.; Floros, J. Cap-independent translation of human SP-A 5'-UTR variants: A double-loop structure and cis-element contribution. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2009**, *296*, L635–L647. [[CrossRef](#)] [[PubMed](#)]
155. Damiano, F.; Alemanno, S.; Gnoni, G.V.; Siculella, L. Translational control of the sterol-regulatory transcription factor SREBP-1 mRNA in response to serum starvation or ER stress is mediated by an internal ribosome entry site. *Biochem. J.* **2010**, *429*, 603–612. [[CrossRef](#)]
156. Grainger, L.; Cicchini, L.; Rak, M.; Petrucelli, A.; Fitzgerald, K.D.; Semler, B.L.; Goodrum, F. Stress-Inducible Alternative Translation Initiation of Human Cytomegalovirus Latency Protein pUL. *J. Virol.* **2010**, *84*, 9472–9486. [[CrossRef](#)]
157. Kanda, T.; Imazeki, F.; Nakamoto, S.; Okitsu, K.; Fujiwara, K.; Yokosuka, O. Internal ribosomal entry-site activities of clinical iso-late-derived hepatitis A virus and inhibitory effects of amantadine. *Hepatol. Res.* **2010**, *40*, 415–423. [[CrossRef](#)]
158. Miura, P.; Coriati, A.; Bélanger, G.; De Repentigny, Y.; Lee, J.; Kothary, R.; Holcik, M.; Jasmin, B. The utrophin A 5'-UTR drives cap-independent translation exclusively in skeletal muscles of transgenic mice and interacts with eEF1A. *Hum. Mol. Genet.* **2010**, *19*, 1211–1220. [[CrossRef](#)]
159. Saffran, H.A.; Read, G.S.; Smiley, J.R. Evidence for Translational Regulation by the Herpes Simplex Virus Virion Host Shutoff Protein. *J. Virol.* **2010**, *84*, 6041–6049. [[CrossRef](#)]
160. Vallejos, M.; Ramdohr, P.; Valiente-Echeverría, F.; Tapia, K.; Rodriguez, F.E.; Lowy, F.; Huidobro-Toro, J.P.; Dangerfield, J.A.; López-Lastra, M. The 5'-untranslated region of the mouse mammary tumor virus mRNA exhibits cap-independent translation initiation. *Nucleic Acids Res.* **2009**, *38*, 618–632. [[CrossRef](#)]
161. Licursi, M.; Christian, S.L.; Pongnopparat, T.; Hirasawa, K. In vitro and in vivo comparison of viral and cellular internal ribosome entry sites for bicistronic vector expression. *Gene Ther.* **2011**, *18*, 631–636. [[CrossRef](#)]

162. Mahon, M.J. Vectors bicistronically linking a gene of interest to the SV40 large T antigen in combination with the SV40 origin of replication enhance transient protein expression and luciferase reporter activity. *Biotech.* **2011**, *51*, 119–128. [[CrossRef](#)] [[PubMed](#)]
163. Martin, J.; Masri, J.; Cloninger, C.; Holmes, B.; Artinian, N.; Funk, A.; Ruegg, T.; Anderson, L.; Bashir, T.; Bernath, A.; et al. Phosphomimetic Substitution of Heterogeneous Nuclear Ribonucleoprotein A1 at Serine 199 Abolishes AKT-dependent Internal Ribosome Entry Site-transacting Factor (ITAF) Function via Effects on Strand Annealing and Results in Mammalian Target of Rapamycin Complex 1 (mTORC1) Inhibitor Sensitivity. *J. Biol. Chem.* **2011**, *286*, 16402–16413. [[CrossRef](#)] [[PubMed](#)]
164. Shi, G.; Yagyu, F.; Shimizu, Y.; Shimizu, K.; Oshima, M.; Iwamoto, A.; Gao, B.; Liu, W.; Gao, G.F.; Kitamura, Y. Flow cytometric assay using two fluorescent proteins for the function of the internal ribosome entry site of hepatitis C virus. *Cytom. Part A* **2011**, *79*, 653–660. [[CrossRef](#)] [[PubMed](#)]
165. Bahar Halpern, K.; Veprik, A.; Rubins, N.; Naaman, O.; Walker, M.D. GPR41 gene expression is mediated by internal ribosome entry site (IRES)-dependent translation of bicistronic mRNA encoding GPR40 and GPR41 proteins. *J. Biol. Chem.* **2012**, *287*, 20154–20163. [[CrossRef](#)]
166. Friis, M.B.; Rasmussen, T.B.; Belsham, G.J. Modulation of Translation Initiation Efficiency in Classical Swine Fever Virus. *J. Virol.* **2012**, *86*, 8681–8692. [[CrossRef](#)]
167. Jiang, H.; Schwertz, H.; Schmid, D.I.; Jones, B.B.; Kriesel, J.; Martinez, M.L.; Kraiss, L.W. Different mechanisms preserve translation of pro-programmed cell death 8 and JunB in virus-infected endothelial cells. *Arter. Thromb. Vasc. Biol.* **2012**, *32*, 997–1004. [[CrossRef](#)]
168. Kanda, T.; Wu, S.; Kiyohara, T.; Nakamoto, S.; Jiang, X.; Miyamura, T.; Imazeki, F.; Ishii, K.; Wakita, T.; Yokosuka, O. Interleukin-29 Suppresses Hepatitis A and C Viral Internal Ribosomal Entry Site-Mediated Translation. *Viral Immunol.* **2012**, *25*, 379–386. [[CrossRef](#)]
169. Lemp, N.A.; Hiraoka, K.; Kasahara, N.; Logg, C.R. Cryptic transcripts from a ubiquitous plasmid origin of replication confound tests for cis-regulatory function. *Nucleic Acids Res.* **2012**, *40*, 7280–7290. [[CrossRef](#)]
170. Li, M.; Man, N.; Qiu, H.; Cai, S.; He, X.; He, X.; Lu, X. Detection of an internal translation activity in the 5' region of Bombyx mori infectious flacherie virus. *Appl. Microbiol. Biotechnol.* **2012**, *95*, 697–705. [[CrossRef](#)]
171. Licursi, M.; Komatsu, Y.; Pongnopparat, T.; Hirasawa, K. Promotion of viral internal ribosomal entry site-mediated translation under amino acid starvation. *J. Gen. Virol.* **2012**, *93*, 951–962. [[CrossRef](#)]
172. Mansha, M.; Wasim, M.; Ploner, C.; Hussain, A.; Latif, A.A.; Tariq, M.; Kofler, A. Problems encountered in bicistronic IRES-GFP expression vectors employed in functional analyses of GC-induced genes. *Mol. Biol. Rep.* **2012**, *39*, 10227–10234. [[CrossRef](#)]
173. Pacheco, A.; Twiss, J.L. Localized IRES-dependent translation of ER chaperone protein mRNA in sensory axons. *PLoS ONE* **2012**, *7*, e40788.
174. Petz, M.; Them, N.; Huber, H.; Beug, H.; Mikulits, W. La enhances IRES-mediated translation of laminin B1 during malignant epithelial to mesenchymal transition. *Nucleic Acids Res.* **2011**, *40*, 290–302. [[CrossRef](#)]
175. Pichorner, A.; Sack, U.; Kobelt, D.; Kelch, I.; Arlt, F.; Smith, J.; Walther, W.; Schlag, P.M.; Stein, U. In vivo imaging of colorectal cancer growth and metastasis by targeting MACC1 with shRNA in xenografted mice. *Clin. Exp. Metastasis* **2012**, *29*, 573–583. [[CrossRef](#)]
176. RübSamen, D.; Bleses, J.S.; Schulz, K.; Döring, C.; Hansmann, M.-L.; Heide, H.; Weigert, A.; Schmid, T.; Brüne, B. IRES-dependent translation of egr2 is induced under inflammatory conditions. *RNA* **2012**, *18*, 1910–1920. [[CrossRef](#)]
177. Shenfeld, M.; Hachmo, Y.; Frenkel, M.; Dafni, N.; Boettcher, M.; Hoheisel, J.D.; Canaani, D. ER-alpha-cDNA as part of a bicistronic transcript gives rise to high frequency, long term, receptor expressing cell clones. *PLoS ONE* **2012**, *7*, e31977. [[CrossRef](#)]
178. Vargas, J.E.; Salton, G.; Laino, A.S.D.C.; Pires, T.D.; Bonamino, M.; Lenz, G.; Delgado-Cañedo, A. pLR: A lentiviral backbone series to stable transduction of bicistronic genes and exchange of promoters. *Plasmid* **2012**, *68*, 179–185. [[CrossRef](#)]
179. Villaflores, O.B.; Chen, Y.J.; Chen, C.P.; Yeh, J.M.; Wu, T.Y. Effects of curcumin and demethoxycurcumin on amyloid- β precursor and tau proteins through the internal ribosome entry sites: A potential therapeutic for Alzheimer's disease. *Taiwan. J. Obstet. Gynecol.* **2012**, *51*, 554–564. [[CrossRef](#)] [[PubMed](#)]
180. Wang, Q.; Liu, Y.; An, D.; Diao, H.; Xu, W.; He, X.; Sun, R.; Wei, L.; Li, L. Regulation of hepatitis C virus translation initiation by iron: Role of eIF3 and La protein. *Virus Res.* **2012**, *167*, 302–309. [[CrossRef](#)] [[PubMed](#)]
181. Carbone, D.; Vignard, V.; Séhédic, D.; Moreau-Aubry, A.; Florenceau, L.; Charpentier, M.; Mikulits, W.; Labarrière, N.; Lang, F. The Melanoma Antigens MELOE-1 and MELOE-2 Are Translated from a Bona Fide Polycistronic mRNA Containing Functional IRES Sequences. *PLoS ONE* **2013**, *8*, e75233. [[CrossRef](#)] [[PubMed](#)]
182. Khan, D.; Sharathchandra, A.; Ponnuswamy, A.; Grover, R.; Das, S. Effect of a natural mutation in the 5' untranslated region on the translational control of p53 mRNA. *Oncogene* **2012**, *32*, 4148–4159. [[CrossRef](#)] [[PubMed](#)]
183. Plank, T.D.; Whitehurst, J.T.; Kieft, J.S. Cell type specificity and structural determinants of IRES activity from the 5' leaders of different HIV-1 transcripts. *Nucleic Acids Res.* **2013**, *41*, 6698–6714. [[CrossRef](#)]
184. Shaltouki, A.; Harford, T.J.; Komar, A.A.; Weyman, C.M. IRES-mediated translation of the pro-apoptotic Bcl2 family member PUMA. *Translation* **2013**, *1*, e24391. [[CrossRef](#)]
185. Spatz, S.J.; Volkening, J.D.; Mullis, R.; Li, F.; Mercado, J.; Zsak, L. Expression of chicken parvovirus VP2 in chicken embryo fibroblasts requires codon optimization for production of naked DNA and vectored meagrid herpesvirus type 1 vaccines. *Virus Genes* **2013**, *47*, 259–267. [[CrossRef](#)]
186. Wang, Z.; Wu, L.; Cheng, X.; Liu, S.; Li, B.; Li, H.; Sun, D. Replication-competent infectious hepatitis B virus vectors carrying substantially sized transgenes by redesigned viral polymerase translation. *PLoS ONE* **2013**, *8*, e60306.

187. Cheng, X.; Gao, X.-C.; Wang, J.-P.; Yang, X.-Y.; Wang, Y.; Li, B.-S.; Kang, F.-B.; Li, H.-J.; Nan, Y.-M.; Sun, D.-X. Tricistronic hepatitis C virus subgenomic replicon expressing double transgenes. *World J. Gastroenterol.* **2014**, *20*, 18284–18295. [[CrossRef](#)]
188. Fernández, A.; Guzmán, S.; Cruz, Y.; Zamorano, P. Construction of bicistronic lentiviral vectors for tracking the expression of CDNF in transduced cells. *Plasmid* **2014**, *76*, 15–23. [[CrossRef](#)]
189. Rübsamen, D.; Kunze, M.M.; Buderus, V.; Brauß, T.F.; Bajer, M.M.; Brüne, B.; Schmid, T. Inflammatory Conditions Induce IRES-Dependent Translation of cyp24a. *PLoS ONE* **2014**, *9*, e85314. [[CrossRef](#)]
190. Templin, A.T.; Maier, B.; Tersey, S.A.; Hatanaka, M.; Mirmira, R.G. Maintenance of Pdx1 mRNA translation in islet beta-cells during the unfolded protein response. *Mol. Endocrinol.* **2014**, *28*, 1820–1830. [[CrossRef](#)]
191. Wang, Q.S.; Jan, E. Switch from Cap-to Factorless IRES-Dependent 0 and +1 Frame Translation during Cellular Stress and Dicitrovirus Infection. *PLoS ONE* **2014**, *9*, e103601. [[CrossRef](#)]
192. Weingarten-Gabbay, S.; Khan, D.; Liberman, N.; Yoffe, Y.; Bialik, S.; Das, S.; Oren, M.; Kimchi, A. The translation initiation factor DAP5 promotes IRES-driven translation of p53 mRNA. *Oncogene* **2013**, *33*, 611–618. [[CrossRef](#)]
193. Bisio, A.; Latorre, E.; Andreotti, V.; Paillerets, B.B.-D.; Harland, M.; Scarra, G.B.; Ghiorzo, P.; Spitale, R.C.; Provenzani, A.; Inga, A. The 5'-untranslated region of p16INK4a melanoma tumor suppressor acts as a cellular IRES, controlling mRNA translation under hypoxia through YBX1 binding. *Oncotarget* **2015**, *6*, 39980–39994. [[CrossRef](#)]
194. Chung, Y.-C.; Hsieh, F.-C.; Lin, Y.-J.; Wu, T.-Y.; Lin, C.-W.; Lin, C.-T.; Tang, N.-Y.; Jinn, T.-R. Magnesium lithospermate B and rosmarinic acid, two compounds present in *Salvia miltiorrhiza*, have potent antiviral activity against enterovirus 71 infections. *Eur. J. Pharmacol.* **2015**, *755*, 127–133. [[CrossRef](#)]
195. Dai, W.; Ma, W.; Li, Q.; Tao, Y.; Ding, P.; Zhu, R.; Jin, J. The 5'-UTR of DDB2 harbors an IRES element and upregulates translation during stress conditions. *Gene* **2015**, *573*, 57–63. [[CrossRef](#)]
196. Khan, D.; Katoch, A.; Das, A.; Sharathchandra, A.; Lal, R.; Roy, P.; Das, S.; Chattopadhyay, S. Reversible induction of translational isoforms of p53 in glucose deprivation. *Cell Death Differ.* **2015**, *22*, 1203–1218. [[CrossRef](#)]
197. Lang, L.; Ding, H.-F.; Chen, X.; Sun, S.-Y.; Liu, G.; Yan, C. Internal Ribosome Entry Site-Based Bicistronic In Situ Reporter Assays for Discovery of Transcription-Targeted Lead Compounds. *Chem. Biol.* **2015**, *22*, 957–964. [[CrossRef](#)]
198. Licursi, M.; Carmona-Martinez, R.A.; Razavi, S.; Hirasawa, K. Promotion of Viral IRES-Mediated Translation Initiation under Mild Hypothermia. *PLoS ONE* **2015**, *10*, e0126174. [[CrossRef](#)]
199. Vaklavas, C.; Meng, Z.; Choi, H.; Grizzle, W.E.; Zinn, K.R.; Blume, S.W. Small molecule inhibitors of IRES-mediated translation. *Cancer Biol. Ther.* **2015**, *16*, 1471–1485. [[CrossRef](#)]
200. Andreotti, V.; Bisio, A.; Paillerets, B.B.-D.; Harland, M.; Cabaret, O.; Newton-Bishop, J.; Pastorino, L.; Bruno, W.; Bertorelli, R.; De Sanctis, V.; et al. The CDKN2A/p16INK4a 5'UTR sequence and translational regulation: Impact of novel variants predisposing to melanoma. *Pigment. Cell Melanoma Res.* **2015**, *29*, 210–221. [[CrossRef](#)]
201. Chan, S.W. Hydrogen peroxide induces La cytoplasmic shuttling and increases hepatitis C virus internal ribosome entry site-dependent translation. *J. Gen. Virol.* **2016**, *97*, 2301–2315. [[CrossRef](#)]
202. Kanda, T.; Ozawa, M.; Tsukiyama-Kohara, K. IRES-mediated translation of foot-and-mouth disease virus (FMDV) in cultured cells derived from FMDV-susceptible and -insusceptible animals. *BMC Vet Res.* **2016**, *12*, 66. [[CrossRef](#)]
203. Loundras, E.A.; Herod, M.R.; Harris, M.; Stonehouse, N.J. Foot-and-mouth disease virus genome replication is unaffected by inhibition of type III phosphatidylinositol-4-kinases. *J. Gen. Virol.* **2016**, *97*, 2221–2230. [[CrossRef](#)] [[PubMed](#)]
204. Ma, X.X.; Feng, Y.P.; Gu, Y.X.; Zhou, J.H.; Ma, Z.R. Effect of the nucleotides surrounding the start codon on the translation of foot-and-mouth disease virus RNA. *Acta Virol.* **2016**, *60*, 151–155. [[CrossRef](#)]
205. Rivas-Aravena, A.; Muñoz, P.; Jorquera, P.; Diaz, A.; Reinoso, C.; González-Catrilébun, S.; Sandino, A.M. Study of RNA-A Initiation Translation of The Infectious Pancreatic Necrosis Virus. *Virus Res.* **2017**, *240*, 121–129. [[CrossRef](#)] [[PubMed](#)]
206. Badawi, A.; Biyanee, A.; Nasrullah, U.; Winslow, S.; Schmid, T.; Pfeilschifter, J.; Eberhardt, W. Inhibition of IRES-dependent translation of caspase-2 by HuR confers chemotherapeutic drug resistance in colon carcinoma cells. *Oncotarget* **2018**, *9*, 18367–18385. [[CrossRef](#)] [[PubMed](#)]
207. Damiano, F.; Testini, M.; Tocci, R.; Gnoni, G.V.; Siculella, L. Translational control of human acetyl-CoA carboxylase 1 mRNA is mediated by an internal ribosome entry site in response to ER stress, serum deprivation or hypoxia mimetic CoCl₂. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2018**, *1863*, 388–398. [[CrossRef](#)] [[PubMed](#)]
208. Deng, Z.; Zhang, S.; Gu, S.; Ni, X.; Zeng, W.; Li, X. Useful Bicistronic Reporter System for Studying Poly(A) Site-Defining cis Elements and Regulation of Alternative Polyadenylation. *Int. J. Mol. Sci.* **2018**, *19*, 279. [[CrossRef](#)] [[PubMed](#)]
209. Al-Zeer, M.A.; Dutkiewicz, M.; von Hacht, A.; Kreuzmann, D.; Rohrs, V.; Kurreck, J. Alternatively spliced variants of the 5'-UTR of the ARPC2 mRNA regulate translation by an internal ribosome entry site (IRES) harboring a guanine-quadruplex motif. *RNA Biol.* **2019**, *16*, 1622–1632. [[CrossRef](#)]
210. Lee, S.; Kim, J.-A.; Kim, H.-D.; Chung, S.; Kim, K.; Choe, H.K. Real-Time Temporal Dynamics of Bicistronic Expression Mediated by Internal Ribosome Entry Site and 2A Cleaving Sequence. *Mol. Cells* **2019**, *42*, 418–425. [[CrossRef](#)]
211. Matsui, T.; Handa, Y.; Kanda, T.; Tsukiyama-Kohara, K. Silencing of the foot-and-mouth disease virus internal ribosomal entry site by targeting relatively conserved region among serotypes. *Virus Genes* **2019**, *55*, 786–794. [[CrossRef](#)]
212. Koirala, D.; Lewicka, A.; Koldobskaya, Y.; Huang, H.; Piccirilli, J.A. Synthetic Antibody Binding to a Preorganized RNA Domain of Hepatitis C Virus Internal Ribosome Entry Site Inhibits Translation. *ACS Chem. Biol.* **2020**, *15*, 205–216. [[CrossRef](#)]

213. Quilici, L.S.; Pereira, I.S.; Andrade, A.C.; Albuquerque, F.C.; Brigido, M.M.; Maranhão, A.Q. A minimal cytomegalovirus intron A variant can improve transgene expression in different mammalian cell lines. *Biotechnol. Lett.* **2012**, *35*, 21–27. [[CrossRef](#)]
214. Stoneley, M.; Subkhankulova, T.; Le Quesne, J.P.; Coldwell, M.J.; Jopling, C.L.; Belsham, G.J.; Willis, A.E. Analysis of the c-myc IRES; a potential role for cell-type specific trans-acting factors and the nuclear compartment. *Nucleic Acids Res.* **2000**, *28*, 687–694. [[CrossRef](#)]
215. Shiroki, K.; Ohsawa, C.; Sugi, N.; Wakiyama, M.; Miura, K.I.; Watanabe, M.; Sugano, S. Internal ribosome entry site-mediated translation of Smad5 in vivo: Requirement for a nuclear event. *Nucleic Acids Res.* **2002**, *30*, 2851–2861. [[CrossRef](#)]
216. Ainaoui, N.; Hantelys, F.; Renaud-Gabardos, E.; Bunel, M.; Lopez, F.; Pujol, F.; Planes, R.; Bahraoui, E.; Pichereaux, C.; Burlet-Schiltz, O.; et al. Promoter-Dependent Translation Controlled by p54nrb and hnRNPM during Myoblast Differentiation. *PLoS ONE* **2015**, *10*, e0136466. [[CrossRef](#)]
217. Sadowski, I.; Ma, J.; Triezenberg, S.; Ptashne, M. GAL4-VP16 is an unusually potent transcriptional activator. *Nat. Cell Biol.* **1988**, *335*, 563–564. [[CrossRef](#)]
218. Graham, F.; Van Der Eb, A. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **1973**, *52*, 456–467. [[CrossRef](#)]
219. Neumann, E.; Schaefer-Ridder, M.; Wang, Y.; Hofschneider, P.H. Gene transfer into mouse lymphoma cells by electroporation in high electric fields. *EMBO J.* **1982**, *1*, 841–845. [[CrossRef](#)]
220. Felgner, P.L.; Gadek, T.R.; Holm, M.; Roman, R.; Chan, H.W.; Wenz, M.; Northrop, J.P.; Ringold, G.M.; Danielsen, M. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 7413–7417. [[CrossRef](#)]
221. Boussif, O.; Lezoualc'h, F.; Zanta, M.A.; Mergny, M.D.; Scherman, D.; Demeneix, B.; Behr, J.P. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 7297–7301. [[CrossRef](#)]
222. Balleza, E.; Kim, J.M.; Cluzel, P. Systematic characterization of maturation time of fluorescent proteins in living cells. *Nat. Methods* **2018**, *15*, 47–51. [[CrossRef](#)]
223. Chen, P.H.; White, C.E. Comparison of rectal, microchip transponder, and infrared thermometry techniques for obtaining body temperature in the laboratory rabbit (*Oryctolagus cuniculus*). *J. Am. Assoc. Lab. Anim. Sci.* **2006**, *45*, 57–63. [[PubMed](#)]
224. Jackson, R.; Hunt, T. Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. *Methods Enzymol.* **1983**, *96*, 50–57. [[PubMed](#)]
225. Rose, J.K.; Trachsel, H.; Leong, K.; Baltimore, D. Inhibition of translation by poliovirus: Inactivation of a specific initiation factor. *Proc. Natl. Acad. Sci. USA* **1978**, *75*, 2732–2736. [[CrossRef](#)] [[PubMed](#)]
226. Belsham, G.J.; Sonenberg, N. RNA-protein interactions in regulation of picornavirus RNA translation. *Microbiol. Rev.* **1996**, *60*, 499–511. [[CrossRef](#)] [[PubMed](#)]
227. Sulima, S.O.; Dinman, J.D. The Expanding Riboverse. *Cells* **2019**, *8*, 1205. [[CrossRef](#)] [[PubMed](#)]
228. Ohlmann, T.; Prévôt, D.; Décimo, D.; Roux, F.; Garin, J.; Morley, S.J.; Darlix, J.-L. In Vitro Cleavage of eIF4GI but not eIF4GII by HIV-1 Protease and its Effects on Translation in the Rabbit Reticulocyte Lysate System. *J. Mol. Biol.* **2002**, *318*, 9–20. [[CrossRef](#)]
229. Soto Rifo, R.; Ricci, E.P.; Décimo, D.; Moncorgé, O.; Ohlmann, T. Back to basics: The untreated rabbit reticulocyte lysate as a competitive system to recapitulate cap/poly(A) synergy and the selective advantage of IRES-driven translation. *Nucleic Acids Res.* **2007**, *35*, e121. [[CrossRef](#)]
230. Yoon, A.; Peng, G.; Brandenburger, Y.; Zollo, O.; Xu, W.; Rego, E.; Ruggero, D. Impaired Control of IRES-Mediated Translation in X-Linked Dyskeratosis Congenita. *Science* **2006**, *312*, 902–906. [[CrossRef](#)]
231. Barna, M.; Pusic, A.; Zollo, O.; Costa, M.; Kondrashov, N.; Rego, E.; Rao, P.H.; Ruggero, D. Suppression of Myc oncogenic activity by ribosomal protein haploinsufficiency. *Nat. Cell Biol.* **2008**, *456*, 971–975. [[CrossRef](#)]
232. McNabb, D.S.; Reed, R.; Marciniak, R.A. Dual Luciferase Assay System for Rapid Assessment of Gene Expression in *Saccharomyces cerevisiae*. *Eukaryot. Cell* **2005**, *4*, 1539–1549. [[CrossRef](#)]
233. England, C.G.; Ehlerding, E.B.; Cai, W. NanoLuc: A Small Luciferase Is Brightening Up the Field of Bioluminescence. *Bioconj. Chem.* **2016**, *27*, 1175–1187. [[CrossRef](#)]
234. Liu, B.; Qian, S.B. Translational reprogramming in cellular stress response. *Wiley Interdiscip. Rev. RNA* **2014**, *5*, 301–315. [[CrossRef](#)]
235. Semler, B.L.; Waterman, M.L. IRES-mediated pathways to polysomes: Nuclear versus cytoplasmic routes. *Trends Microbiol.* **2008**, *16*, 1–5. [[CrossRef](#)]
236. Brown, B.A.; Ehrenfeld, E. Translation of poliovirus RNA in vitro: Changes in cleavage pattern and initiation sites by ribosomal salt wash. *Virology* **1979**, *97*, 396–405. [[CrossRef](#)]
237. Subkhankulova, T.; Mitchell, S.A.; Willis, A.E. Internal ribosome entry segment-mediated initiation of c-Myc protein synthesis following genotoxic stress. *Biochem. J.* **2001**, *359*, 183–192. [[CrossRef](#)]
238. Fernandez, J.; Yaman, I.; Mishra, R.; Merrick, W.C.; Snider, M.D.; Lamers, W.H.; Hatzoglou, M. Internal Ribosome Entry Site-mediated Translation of a Mammalian mRNA Is Regulated by Amino Acid Availability. *J. Biol. Chem.* **2001**, *276*, 12285–12291. [[CrossRef](#)]
239. Chen, C.; Sarnow, P. Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs. *Science* **1995**, *268*, 415–417. [[CrossRef](#)]
240. Candeias, M.M.; Powell, D.J.; Roubalova, E.; Apcher, S.; Bourougaa, K.; Vojtesek, B.; Fähræus, R. Expression of p53 and p53/47 are controlled by alternative mechanisms of messenger RNA translation initiation. *Oncogene* **2006**, *25*, 6936–6947. [[CrossRef](#)]

-
241. Zydowicz-Machtel, P.; Swiatkowska, A.; Popenda, Ł.; Gorska, A.; Ciesiołka, J. Variants of the 5'-terminal region of p53 mRNA influence the ribosomal scanning and translation efficiency. *Sci. Rep.* **2018**, *8*, 1533. [[CrossRef](#)]
 242. Thompson, S.R. So you want to know if your message has an IRES? *Wiley Interdiscip. Rev. RNA* **2012**, *3*, 697–705. [[CrossRef](#)]
 243. Moher, D.; Liberati, A.; Tetzlaff, J.; Altman, D.G.; The PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement. *PLoS Med.* **2009**, *6*. [[CrossRef](#)]