Production of human translation-competent lysates using dual centrifugation

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ABSTRACT

Translation is a central process in gene expression and the development of efficient *in vitro* translation systems has been the focus of scientific efforts for many decades. The production of translation-competent lysates originating from human cells or tissues remains challenging, mainly due to the variability of cell lysis conditions. Here we present a robust and fast method based on dual centrifugation that allows the preparation of cytoplasmic extracts from human cells that efficiently translate mRNAs in a capped and IRES-mediated way. We optimized lysate preparation and *in vitro* translation conditions and show that dual centrifugation allows the production of human lysates under detergent-free conditions and is ideally suited to produce ample amounts of human translation-competent lysates.

INTRODUCTION

Cell-free translation is the process of synthesizing proteins in extracts without the use of intact cells. During the last five decades, cell-free extracts have been used to recapitulate key steps of translation and have provided valuable insights into this fundamental process (1). Bacterial-based lysates have been extensively used to develop, understand, and expand the

applications of *in vitro* translation (2). Ranging from the historical experiments that led to the determination of the genetic code (3,4) to recent advances that allow the design of orthogonal *in vitro* translation systems (5,6), cell-free protein synthesis has revolutionized the possibilities of synthetic biology by employing and expanding the properties of *in vitro* translation. Moreover, cell-free systems have allowed the production of numerous recombinant proteins with clear advantages compared to production in living cells. The possibility of genetic manipulation, the incorporation of unnatural amino acids, the tolerance to toxic proteins and the fast production of results in cell-free extracts have extended the limits of synthetic biochemistry and synthetic biology significantly (2).

Even though the production and utilization of human lysates would be particularly appealing, so far, it has not been equally developed. Human cell-derived lysates provide a better environment for the proper folding of human proteins as they facilitate the incorporation of human-specific post-translation modifications. Moreover, they are an appealing system to study translation-related processes in small or high throughput scale settings. However, several key constraints like the cost and technical difficulties in the reproducible production of eukaryotic translation systems compared to prokaryotic have limited their usage in industry and in research (7).

The variable and reduced protein synthesis output are the most important reasons that impede the routine adoption of *in vitro* translation systems (1). To date, the only mammalian *in vitro* translation system that allows cap-dependent translation and is commercially available is the rabbit reticulocyte lysate (RRL) (8). This system has proven to be a valuable source to study several aspects of mammalian translation. However, it is accompanied by unsurpassed caveats due to reticulocytes being special cells regarding their translation activity and regulation. The requirement of complex manipulation of animal tissue, the increased concentration of endogenous globin mRNAs, and the abundance of RNase M or other tissue-specific nucleases (9) are important limitations of this system. Moreover, the RRL cannot be genetically modified to deplete or enrich certain protein factors (10). Previous efforts have advanced the use of mammalian cells to produce translation-competent lysates. Chinese hamster ovary cells (CHO) have been used to develop an *in vitro* translation system that allows the IRES-dependent translation of reporter constructs and has been shown to produce functional glycosylated proteins of interest (11). The lysates of human origin that support cap-dependent and independent *in vitro* translation of reporter mRNAs originate from HeLa cells (12–14). However, issues concerning the variability of lysis parameters, such as the applied mechanical force, have not been satisfactorily solved.

In our previous efforts to address mechanistic questions about translation termination, we developed an *in vitro* translation system based on HeLa cells that was used to monitor ribosomal density at the termination codon of reporter mRNAs. This protocol was optimized to prepare lysates using the shearing forces of syringe treatment under hypotonic conditions to break up the cells and release functionally active molecules that can reconstitute the complex translation process *in vitro* (15), based on a previously reported protocol (14). During this development, we observed that cell lysis is the most crucial step for the success of our *in vitro* system. To further improve the efficiency and the reproducibility of this step, we applied dual centrifugation (DC) as a novel approach to lyse cells. Dual centrifugation differs from regular centrifugation by combining the simultaneous rotation around two axes. As a consequence of this turning around the second axis, the centrifugal acceleration constantly changes, resulting in powerful agitation of the sample material inside the vials (Fig. 1A). Thus, in contrast to conventional centrifugation that separates components, DC leads to efficient sample homogenization (16,17). Adapting this technique as a cell lysis method, we streamlined the production of translation-competent lysates in various batch sizes in a reproducible manner. The resulting lysates allowed us to dissect the role of Nsp1, a potent virulent factor produced during the early steps of infection by SARS-CoV-2 in human cells (18).

Here we provide evidence for the efficient and reproducible production of cytoplasmic cell extracts in large amounts using dual centrifugation. We show that dual centrifugation can be used for routine cell lysis under different buffer compositions, and we investigate the conditions that give rise to lysates that translate reporter mRNAs in a cap- and IRES-dependent manner. We perform a biochemical characterization of the lysates and provide technical details for their production.

MATERIAL AND METHODS

Cell lines and growth conditions

For all experiments, HeLa S3 (Sigma-Aldrich, ECACC, cat. no. 87110901) were cultured in DMEM/F-12 (Gibco, cat. no. 32500-043) containing 10% (v/v) FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (termed DMEM+/+) at 37°C and 5% CO_2 in humid atmosphere. To start a new culture from a cryopreserved stock (stored in 50 % FCS, 40 % DMEM +/+ and 10% DMSO), 1-2x10^6 cells were seeded into a 75 cm^2 flask (TPP, cat. no. 90026). After reaching confluency of approximately 80% as adherent cells, they were trypsinized and transferred into a suspension culture. The suspension cultures were then grown in volumes between 20 ml and 200 ml under constant agitation (Corning flasks, cat. no. 431405 and 431403) within a range of 0.2x10^6 and 1x10^6 cells/ml.

Preparation of translation-competent lysates

Lysates were prepared from S3 HeLa cell cultures grown to a cell density ranging from $0.8x10^6$ to $1x10^6$ cells/ml. Cells were pelleted (200*g*, 4 °C for 5 min), washed twice with cold PBS pH 7.4 and finally resuspended in ice-cold hypotonic lysis buffer (10 mM HEPES pH 7.3, 10 mM K-acetate, 500 µM Mg-acetate, 5 mM DTT and 1× protease inhibitor cocktail (Bimake, cat. no. B14002)) at a final concentration of $2x10^8$ cells/ml in 2 ml screw cap microtubes (Sarstedt, cat. no. 72.693). To lyse the cells in translation-competent conditions we used 33.78 mM HEPES, 63.06 mM potassium acetate, 0.68 mM MgCl2, 54.05 mM KCl, 0.09 mM Aminoacids,13.51 mM Creatine Phosphate and 229.73 ng/ml Creatin kinase. Cells were lysed by dual centrifugation at 500 RPM (unless otherwise stated), −5 °C for 4 min using ZentriMix 380 R system (Hettich AG) with a 3206 rotor with 3209 adapters. Cell lysis was monitored by staining a 1:10 (v/v, f.c.) diluted sample before and after dual centrifugation with 0.2% (v/v, f.c.) Trypan Blue (Thermo Fisher, cat. no. T10282) using a light microscope with EVE Cell counting slides (NanoEnTek, cat. no. EVS-050). The lysate was centrifuged at 13,000 *g*, 4 °C for 10 min and the supernatant was aliquoted, snap-frozen and stored at −80 °C. For subsequent applications, the lysate could be thawed and refrozen multiple times with a minor loss of translation efficiency.

Plasmids and cloning

The p200-6xMS2 plasmid encoding the Rluc reporter used for *in vitro* transcription is described in (18). We created pCRII-p2luc-UAA-A80 by combining *Not*I-*Kpn*I digested pCRII-hRLuc-200bp-(A)80 (15) and a PCR-amplified template from the plasmid p2luc-UAA (19) using the primers EK117 ATATGCGGCCGCATGACTTCGAAAGTTTATGA and EK118 TATAGGTACCTTACAATTTGGACTTTCCGCCCTT and digested with the same restriction enzymes followed by ligation. The ECMV IRES sequence was subcloned into the pCRII dual-luciferase reporter plasmid pCRII-p2luc-UAA-A80 and pT7CFE1 EMCV IRES from Thermo Fisher by infusion cloning using the primers EK200 CCTCGGTGGTGCTAAGCGAATTAATTCCGGTTATTTTCCACCATA and EK201 GTTTTTGGCGTCTTCCATATTATCATCGTGTTTTTCAAAGGAAAACCAC for the insert amplification and EK202 GAAGACGCCAAAAACATAAAGAAAGGCC and EK203 TTAGCACCACCGAGGCCCCGGA for the vector amplification. The derived plasmid was quick-changed using the primer EK204 AAGGAAGCAGTTCCTCTGGAAGCAACTTGAAGACAAACAAC to delete a second *Hind*III restriction site because this enzyme is used for linearization before run-off *in vitro* transcription.

In vitro transcription of reporter mRNAs

Plasmids encoding the luciferase reporter mRNA by a T7 promoter in a pCRII vector were linearized using a restriction site located downstream of the poly(A) sequence in a reaction containing 40 ng/µl DNA (4 µg total) and 0.5 U/µl restriction enzyme (*Hind*III-HF, NEB, cat. no. R3104L) in 1x CutSmart buffer (NEB, cat. no. B7204S). The digest was monitored by running 200 ng of the DNA on a 1% agarose gel in 1x TAE. The rest of the reaction was purified using the MACHEREY-NAGEL NucleoSpin® Gel and PCR Clean-up kit (cat. no. 740609.50). The isolated DNA was den quantified by measuring OD_{260}.

Using the linearized plasmid as a template (up to 4 µg in total and 40 ng/µl f.c.), the reporter mRNA was *in vitro* transcribed in a reaction containing 1x OPTIZYME transcription buffer (Thermo Fisher Scientific, cat. no. BP81161), 1 mM of each ribonucleotide (Thermo Fisher, cat. no. R0481), 1 U/µl RNase inhibitor (Vazyme, cat. no. R301-03), 0.001 U/µl pyrophosphatase (Thermo Fisher Scientific, cat. no. EF0221) and 5 % (v/v) T7 polymerase (custom-made). The mixture was incubated at 37 °C for 1 hour, and then an equal amount of polymerase was added to boost transcription for another 30 min. Subsequently, the template DNA was degraded by treatment with 0.14 U/µl Turbo DNase (Invitrogen, cat. no. AM2238) at 37 °C for 30 min. Finally, the newly transcribed reporter mRNA was isolated using the Monarch® RNA Cleanup Kit (New England Biolabs, cat. no. T2040L), eluted with 1 mM sodium citrate pH 6.4 (Gene Link, cat. no. 40-5014-05), and quantified as mentioned above.

To generate transcripts containing a 7-methylguanylate cap, we used the Vaccinia Capping System from New England Biolabs (cat. no. M2080S) according to the manufacturer's instructions with the addition that the reaction mix was supplemented with 1 U/µl RNase inhibitor (Vazyme, cat. no. R301-03). The resulting capped mRNA was purified, eluted, and quantified as after the transcription reaction.

In vitro translation

S3 lysates prepared in hypotonic lysis buffer were used at a concentration of 8.88 $x10^7$ cell equivalents/ml. A standard 12.5 µl reaction was supplemented to a final concentration of 15 mM HEPES, pH 7.3, 0.3 mM $MgCl_2$, 24 mM KCl, 28 mM K-acetate, 6 mM creatine phosphate (Roche), 102 ng/µl creatine kinase (Roche, cat.no. 10127566001), 0.4 mM amino acid mixture (Promega, cat. no. L4461) and 1 U/µl RNase inhibitor (Vazyme, cat. no. R301-03), unless otherwise stated. Control reactions contained 320 µg/ml puromycin (Santa Cruz Biotechnology, cat.no. 58-58-2). Before the addition of reporter mRNAs, the mixtures were incubated at 33 °C for 5 min. *In vitro* transcribed and capped mRNAs were incubated for 5 min at 65 °C, 15 min at room temperature (RT), and cooled on ice. Reporter mRNAs were added

to the translation reactions at a final concentration of 5 fmol /µl unless otherwise stated. The translation reaction was routinely performed at 33 °C for 50 min and extended up to 4 hours for time-course experiments and stopped by transferring the sample on ice. To monitor the protein synthesis output, the samples were mixed with 50 µl 1× Renilla-Glo substrate in Renilla-Glo assay buffer (Promega, cat. no. E2720) on a white-bottom 96-well plate (Greiner, cat. no. 655073). The plate was incubated at 30 °C for 10 min and the luminescence signal was measured three times using the TECAN infinite M1000 Pro plate reader and plotted on GraphPad (Version 8.4.2). According to the manufacturer's guidelines, dual-luciferase assays were performed using 40 µl of the substrate in the appropriate buffer for a 10 µl *in vitro* translation assay (Promega, cat. no. E1960). Luminescence was measured using TECAN Infinite M1000. To perform *in vitro* translation using lysates prepared in translation-competent buffer, the lysates were directly diluted to 8.88 x10^7 cell equivalents/ml, supplemented with 1 u/µl RNase inhibitor (Vazyme, cat. no. R301-03), and the reaction was performed as described above.

Subcellular fractionation

For the preparation of control cytoplasmic and nuclear lysates, 1x10^7 HeLa S3 cells were harvested and lysed by resuspension in 1 ml buffer 1 (50 mM HEPES pH 7.3, 140 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.5% NP-40, 0.25% Triton X-100, 1x protease inhibitor cocktail (Bimake, cat. no. B14002)) and incubation for 10 min at 4°C rotating. Next, the samples were centrifuged for 500 *g*, 4°C for 5 min, and the supernatant was collected. The same steps were repeated twice with the pellet, and the supernatants were pooled to obtain the cytoplasmic fraction. Next, the pellet was washed by resuspension in 1 ml buffer 2 (10 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1x protease inhibitor cocktail (Bimake, cat. no. B14002)) and incubation for 10 min at 4°C rotating. The nuclei were pelleted by centrifugation at 500 *g*, 4°C for 5 min, resuspended in 500 µl buffer 3 (1 mM EDTA, 0.5 mM EGTA, 10 mM Tris pH 8.0, 10% SDS, 0.1% deoxycholate, 1% Triton X-100, 1x protease inhibitor cocktail (Bimake, cat. no. B14002)) and sonicated with 3 x 10 s pulses at 45% amplitude (Vibra-Cell, cat. no. 75186). Finally, samples were centrifuged at 16'000 *g,* 4°C for 30 min, and the supernatant was collected to obtain the nuclear fraction.

Western blot analysis

To analyze the products generated by DC, samples were collected before lysis by ZentriMix (HeLa S3 cells in hypotonic lysis buffer, whole cells) and after the final centrifugation step (lysate and pellet). The whole-cell and pellet samples were diluted in three volumes of hypotonic lysis buffer and subjected to sonication for 3 x 10 s pulses at 45% amplitude (Vibra-Cell, cat. no. 75186). Proteins were extracted by chloroform-methanol precipitation from the

DC-derived material and the samples deriving from the detergent-based fraction. From each sample, approx. 6.8 µg (quantification by OD_{280}) in 1.5x LDS loading buffer (Invitrogen, cat. no. NP0008) containing 50 mM DTT were run on a 4-12% Bis-Tris NuPAGE gel (Invitrogen, cat. no. WG1401BOX). Transfer onto a nitrocellulose membrane was performed using the iBlot 2 Dry Blotting System (Invitrogen, cat. no. IB21001 and IB23001). Nuclear and cytoplasmic protein markers were then assessed with antibodies against Tyrosine-Tubulin (Sigma T9028, 1:10'000), GAPDH (Santa Cruz Biotechnology sc-25778, 1:1'000), and Histone H3 (Abcam ab1791, 1:8'000).

RESULTS AND DISCUSSION

Dual centrifugation as a method to produce translation-competent lysates

The efficient lysis of eukaryotic cells under well-defined biochemical conditions is a prerequisite for a wide range of biological assays. The lysis process is often challenging, especially when the isolation of active biomolecules is required for downstream applications. While many different protocols have been developed to produce biologically active lysates that can be used for *in vitro* studies, they are often limited in accurately defining the mechanical force applied during lysis. To apply reproducible shearing forces, we opted for dual centrifugation. In this process, the samples undergo a simultaneous rotation around two axes of symmetry (Fig. 1A). The continuous change of centrifugal forces in the sample vials leads to shearing and homogenizing the processed material (16,17). We rationalized that such a mechanical treatment would allow the lysis of cells independently of the buffer conditions. Importantly, this method accurately defines and fine-tunes the lysis parameters by controlling the centrifugation speed while keeping the processed material at a low temperature.

Based on our previously established *in vitro* translation assays (15), we produced translation-competent lysates using DC as a cell lysis method. The steps of our protocol, as illustrated in Fig. 1B, are the following: **1.** Dual centrifugation of cells in hypotonic lysis buffer. **2.** Short centrifugation to remove cell debris and nuclei **3.** Retention of the supernatant, which can be stored frozen for further use. **4.** Supplementation of the lysate with a buffer mixture containing energy sources and amino acids required for translation. **5.** Lysate activation and addition of reporter mRNAs. **6.** *In vitro* translation followed by detection of the protein product or other downstream approaches.

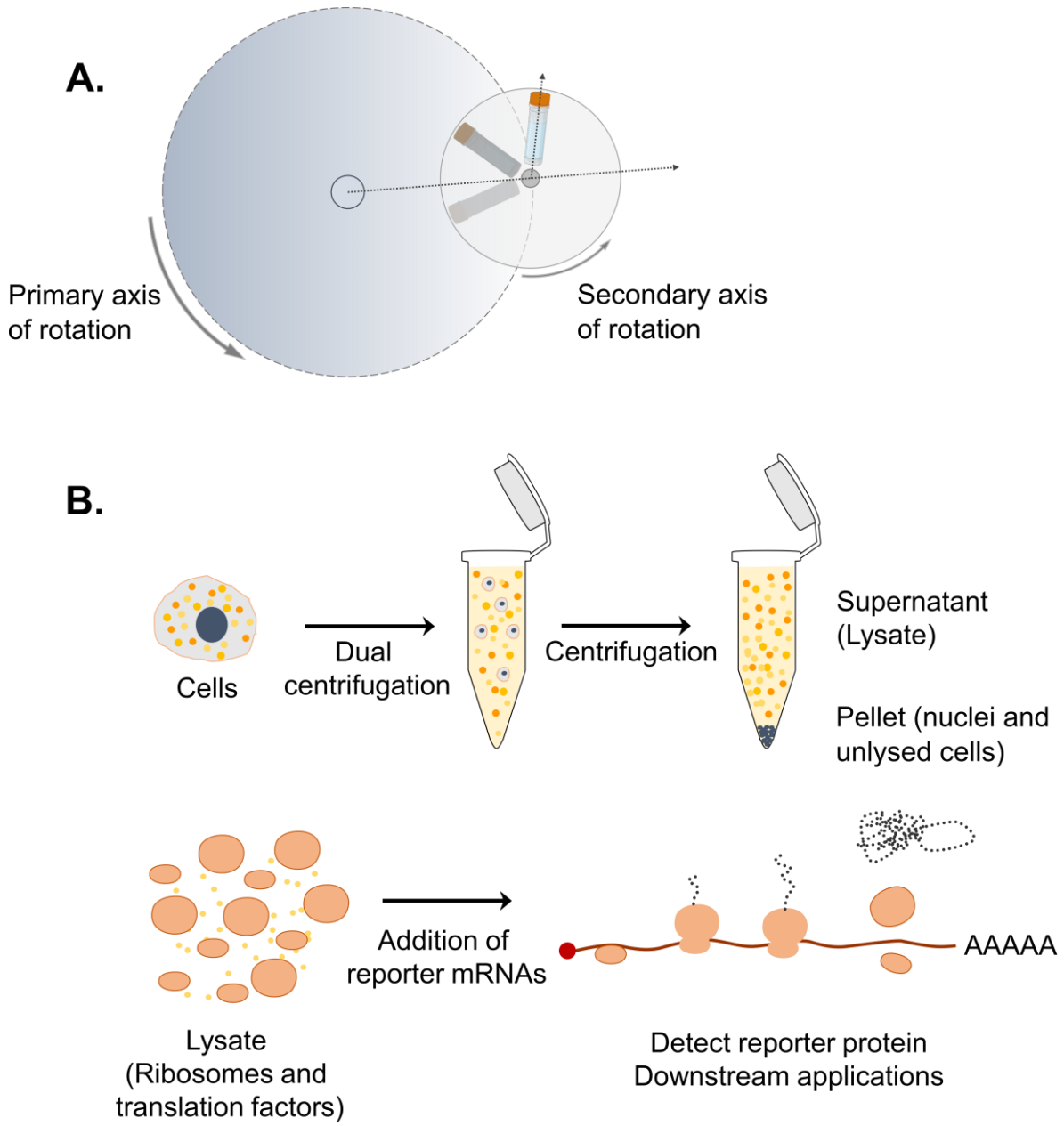

Figure 1: Dual centrifugation as a cell lysis method to produce translation-competent lysates. A. Schematic representation of dual centrifugation. During dual centrifugation, the samples rotate simultaneously around two axes of symmetry. Dashed arrows indicate the direction of centrifugal acceleration. **B.** Graphical overview of the steps that are followed for the production of translation-competent lysates using dual centrifugation.

Dual centrifugation can lyse human cells and allows the isolation of cytoplasmic extracts

Dual centrifugation has not been previously used to disrupt living cells and isolate biologically active lysates. First, we set out to assess whether cell lysis can, in principle, be

performed using this method. We used a hypotonic lysis buffer that is compatible with downstream translation assays, and we applied increasing centrifugal forces to assess the cell lysis efficiency. We established our system using S3 HeLa cells (spinner cells), adapted to grow in suspension culture, and reach high cell populations in small volumes of medium. We harvest cells when they reach a density of 0.8-1.0x10^6 cells/ml in suspension. The lysates are typically prepared in batches of 400-800 µl volume, the cells are washed twice with PBS and resuspended to a hypotonic lysis buffer in a final concentration of 2x10^8 cells/ml. To allow a comparative analysis, the dual centrifugation experiments were performed for 4 minutes, and the temperature of the rotor was set at -5°C, taking into consideration a possible increase of the sample temperature during DC. In all experiments, it was confirmed that the samples did not freeze during this step.

First, we assessed under which centrifugation speed we can achieve cell lysis, ideally without disrupting the cell nuclei. To visualize the lysis efficiency, we performed a Trypan Blue stain that assesses cell membrane integrity and allows us to distinguish living from dead cells; the living cells are visible as white spheres, whereas dead cells and nuclei as dark blue foci. As shown in Fig. 2A, the lysis efficiency increased by applying increased centrifugal force. Lysis was achieved even under isotonic buffer conditions (in PBS, data not shown), rendering dual centrifugation an appealing method for lysing cells in virtually any buffer conditions without adding any detergents. This fact would be of interest in cases that preserving intact, functional biomolecules is crucial. Since we wanted to obtain translation-competent lysates, we opted for a mainly cytoplasmic extract, and we applied milder dual centrifugation forces in the range of 250-1000 RPM to titrate the translation efficiency of the corresponding lysates.

After the dual centrifugation step, we removed the nuclei and insoluble fragments from the lysate by regular centrifugation. To assess whether our lysates are enriched for cytoplasmic components, we assessed cytoplasmic and nuclear markers by immunoblot. We compared our lysates (supernatant after the DC using 500 RPM and regular centrifugation, as shown in Fig. 1B) with total cell lysates from the same cell equivalents and cytoplasmic and nuclear lysates that were obtained using a detergent-based protocol for subcellular fractionation. As shown in Fig. 2B, the supernatant is enriched for cytoplasmic markers (Tyrosine-Tubulin and GAPDH). No signal for histones could be detected even after loading the triple amount of cytoplasmic cell equivalents compared to the nucleus. The pellet contains both nuclei and cytoplasmic components due to the incomplete cell lysis under mild conditions. These results show that dual centrifugation allows the production of lysates that are highly enriched for cytoplasmic components. Further optimization could allow the application of the method to separate cytoplasmic from nuclear fractions.

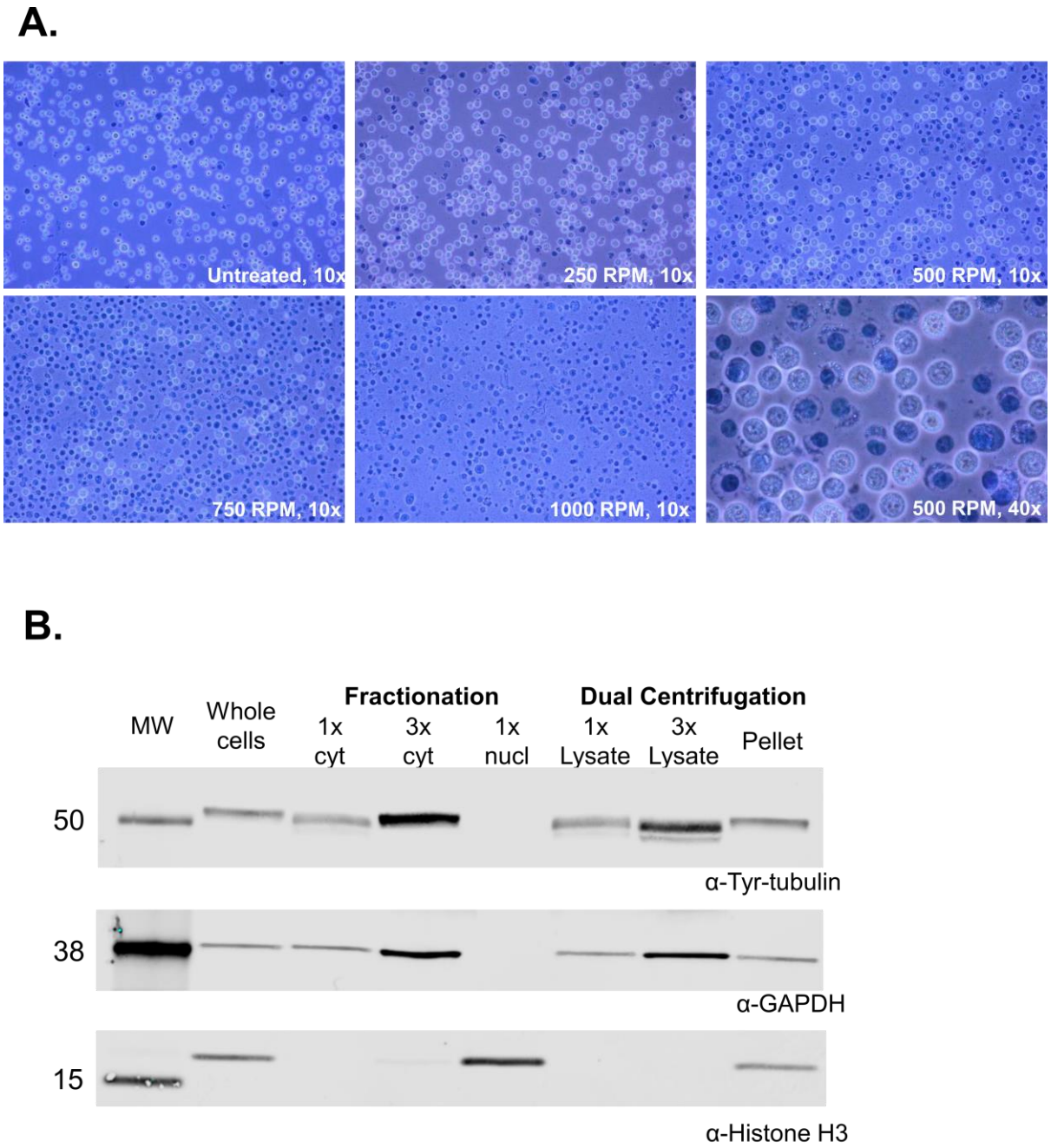

Figure 2: Dual centrifugation allows the production of cytoplasm-enriched lysates. A. Lysis efficiency was assessed by Trypan Blue stain under the light microscope at 10x or 40x magnification. **B.** Western blot analysis of cytoplasmic and nuclear markers of the supernatant (lysate) and the pellet of DC-treated cells. Cytoplasmic and nuclear fractions were prepared using an alternative method of biochemical fractionation. All samples correspond to 6.8 µg protein.

Optimization of the conditions for *in vitro* translation using dual centrifugation-derived lysates

To assess whether the dual centrifugation-derived lysates can translate *in vitro* reporter mRNAs, we used an *in vitro* transcribed and capped *Renilla* luciferase reporter that contains an 80-nucleotides-long poly(A) tail (reporter RLuc).

We set up *in vitro* translation reactions for lysates treated using 250, 500, 750, or 1000 RPM, and we mixed the processed lysates (i.e., the supernatant after the final regular centrifugation) with an equal amount of mRNA for the four reactions. After a 50-min incubation at 33 °C, we measured the activity of RLuc, which documents the translation competence of the lysates. As shown in Fig. 3A, the luminescence measurements verify the translation potential of all the tested lysates, but with an optimal activity of lysates that were treated with 500 RPM and underwent, as shown in Fig. 2A, a relatively mild lysis process. No translation activity was detected in the presence of the translation inhibitor puromycin. The fact that milder lysis conditions lead to a higher translation output may be attributed to the optimal balance between disruption of sensitive components due to the mechanical forces and the efficiency of releasing cytoplasmic components. Additionally, the prevention of lysis of nuclei may also contribute to the higher translation efficiency of the lysates. For these reasons, we adopted these lysis conditions as the standard workflow for the production of translation-competent lysates.

We applied the *in vitro* translation conditions as they were previously optimized for our syringe-treated lysates (15). Among the most crucial factors for efficient translation is the Mg^{2+} concentration. Thus, we decided to titrate this parameter to ensure that the concentration is optimal under the new lysis conditions. Setting as reference a concentration of 0.30 mM, we assessed the efficiency of translation under different $MgCl_2$ concentrations in the translation buffer, and we observed that the concentration of choice falls in the optimal range of translation activity (Fig. 3B). For this reason, we selected this concentration in our *in vitro* experiments. Notably, the range of suitable concentration was wider than for our previous translation system, indicating that lysates prepared by DC are more robust towards small changes of *in vitro* translation conditions.

Next, we titrated the effect of the reporter mRNA concentration in the translation output of the *in vitro* translation reaction. We added increasing amounts of mRNA, reaching a concentration range from 0 to 80 fmol/µl of reaction, and as shown in Fig. 3C, we observed a concentration-dependent increase of the reporter protein output. This increase is linear for concentrations up to 40 fmol/µl (Fig. 3C). Based on this result, we routinely use a concentration of 5 fmol/µl for a standard translation reaction since it corresponds clearly to the linear range

of the response and therefore does not saturate the translation system with mRNA. The concentration of the amount of mRNA used can be adjusted depending on the downstream applications and depends on the translation efficiency of the used mRNAs.

To assess how the duration of the incubation affects the production of the recombinant protein and, therefore, the translation efficiency of the system, we performed a time course from 0 to 150 min (Fig. 3D) and 0 to 4 hours (Fig. 3E). We observed an almost linear increase of the signal during the entire 150-minute incubation. When we prolonged the assay duration, the reporter protein production reached a plateau after 2 hours of translation. Interestingly, there is a non-linear increase of the signal during the first 30 minutes of incubation, implying that the system is not functioning at optimum conditions during this initial phase.

Based on the above experiments, we opted for selecting as standard conditions for optimal translation reaction conditions a $MgCl_2$ concentration of 0.30 mM, using 5 fmol/µl of capped and polyadenylated mRNA for 50 minutes. All the above experiments were performed at 33°C.

Direct cell lysis in a translation-competent buffer

The lysates described above were prepared using a hypotonic lysis buffer and we complement the reaction with the necessary salts, amino acids and energy regeneration components to perform *in vitro* translation. We decided to assess whether we can prepare cell lysates in a buffer that already contains all these translation components. When we lysed the cells directly in such a translation-competent buffer, we obtained translationally active lysates with a similar activity compared to the previous method of lysate preparation (Fig. 3F). This option can improve reproducibility by reducing intermediate steps and would allow the production of more concentrated lysates. Such a lysate would, for example, be ideal for structural studies that require translationally active lysates.

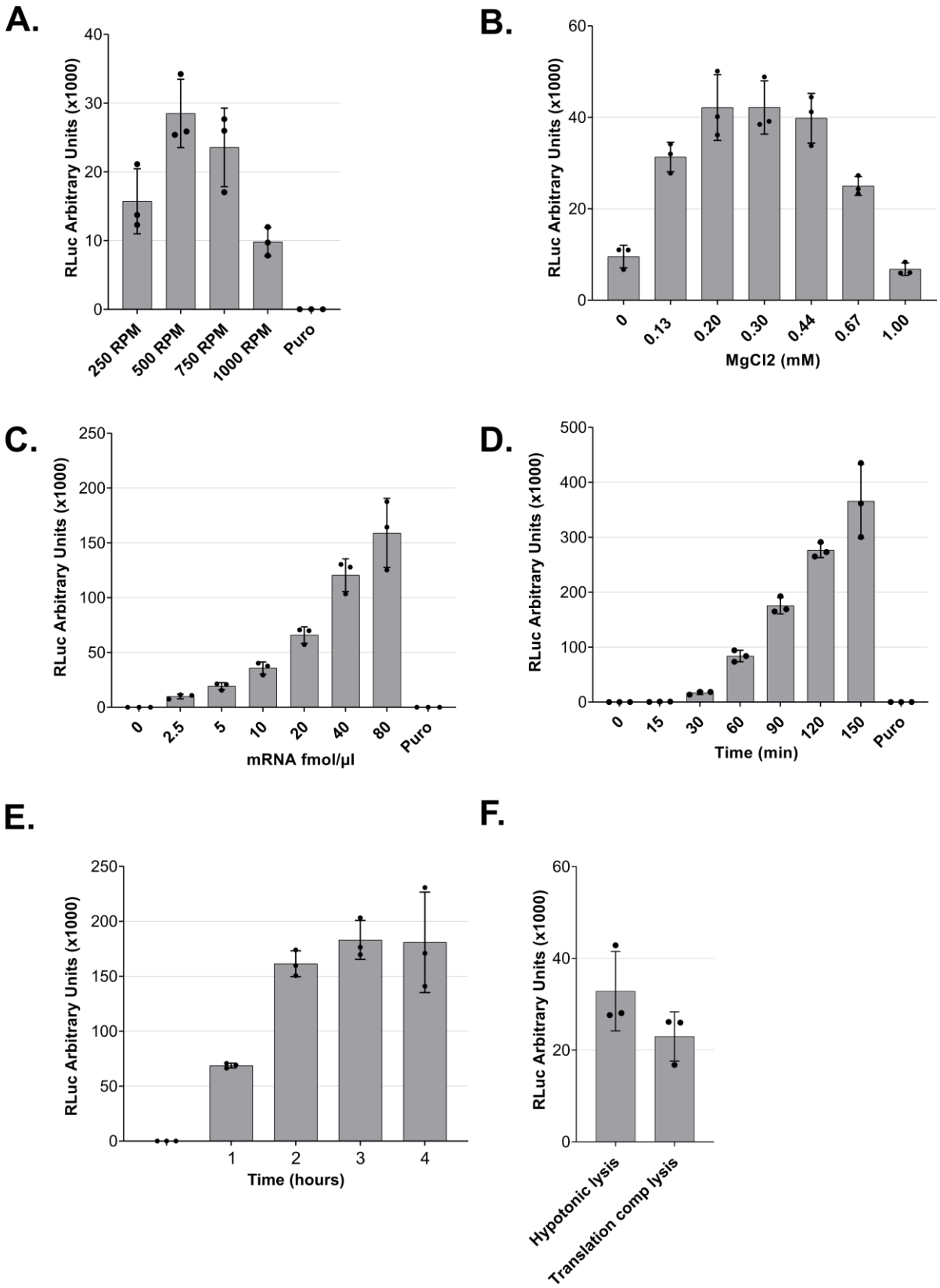

Figure 3: A. *In vitro* translation using lysates that were produced under different DC conditions. **B.** Mg^{2+} titration for *in vitro* translation reactions using 5 fmol/µl RLuc reporter mRNA as substrate. **C.** *In vitro* translation using different mRNA concentrations. **D and E.** Time-course of RLuc *in vitro* translation reactions using 5 fmol/µl. **F.** Comparison of lysates produced using

a hypotonic lysis buffer complemented with components required for *in vitro* translation (hypotonic lysis) versus lysis in a translation-competent buffer (translation comp lysis). For all panels, Rluc activity measurements are depicted as arbitrary units (AU) of luminescence. Each dot depicts the value of an individual experiment and *in vitro* translation (biological replicate) that was measured three times (technical replicates). Mean values and SD are shown.

Dual centrifugation-derived lysates can be used for capped and IRES-dependent *in vitro* translation

Cap-dependent translation is the primary mechanism of translation initiation for mammalian mRNAs. For this reason, we decided to address the effect of a 5´cap on the translation efficiency of mRNA reporters in the *in vitro* translation system by comparing the Rluc output of *in vitro* capped and uncapped mRNAs Renilla luciferase reporter mRNAs. We used equimolar amounts of the two mRNA molecules using a concentration of 5 fmol/μl, and as shown in Fig. 4A, we observed a >30-fold increase in the efficiency of translation in the presence of a 5´cap on the reporter mRNAs. This result provides evidence that our system is sensitive to the presence of a cap, in accordance with the cap-dependent translation initiation of most physiological mRNAs in mammalian cells.

After optimizing the translation efficiency of capped-dependent translation of our lysates, we addressed whether DC-derived lysates can perform Internal Ribosome Entry Site (IRES)-dependent translation, a cap-independent process (20). IRES usage is of interest because many vectors have been developed for *in vitro* protein production employing this principle. To address whether we can perform IRES-dependent translation in our lysates, we prepared a dual-luciferase reporter mRNA encoding *Renilla* and Firefly Luciferase. We used two versions of this construct, one where the two ORFs are separated by an IRES originating from the human EMCV virus and a control version that lacks the IRES sequence (21). The production of a clear Firefly luciferase signal only in the presence of the IRES signifies the capacity of the lysate to perform IRES-dependent translation (Fig. 4B).

After optimizing the translation conditions of dual centrifugation-treated lysates, we compared the efficiency of our system to a commercially available rabbit reticulocyte lysate system (RRL). The translation reaction was performed using three different lysates, prepared under optimal conditions (biological replicates), and every measurement was performed three times. The *in vitro* translation reaction for the RRL was performed according to the manufacturer's guidelines for 50 minutes with an equal amount of Rluc reporter. As shown in Fig. 4C, we observed a more than a four-fold increase of the translation output in all three replicates of our lysates than the reactions where RRL was used. This fact shows that our

system allows the reproducible production of translation-competent lysates with high translation potential.

Overall, our results show that dual centrifugation can be the method of choice to lyse human cells in a buffer-independent manner and produce translation-competent lysates that show a robust cap-dependent and independent activity.

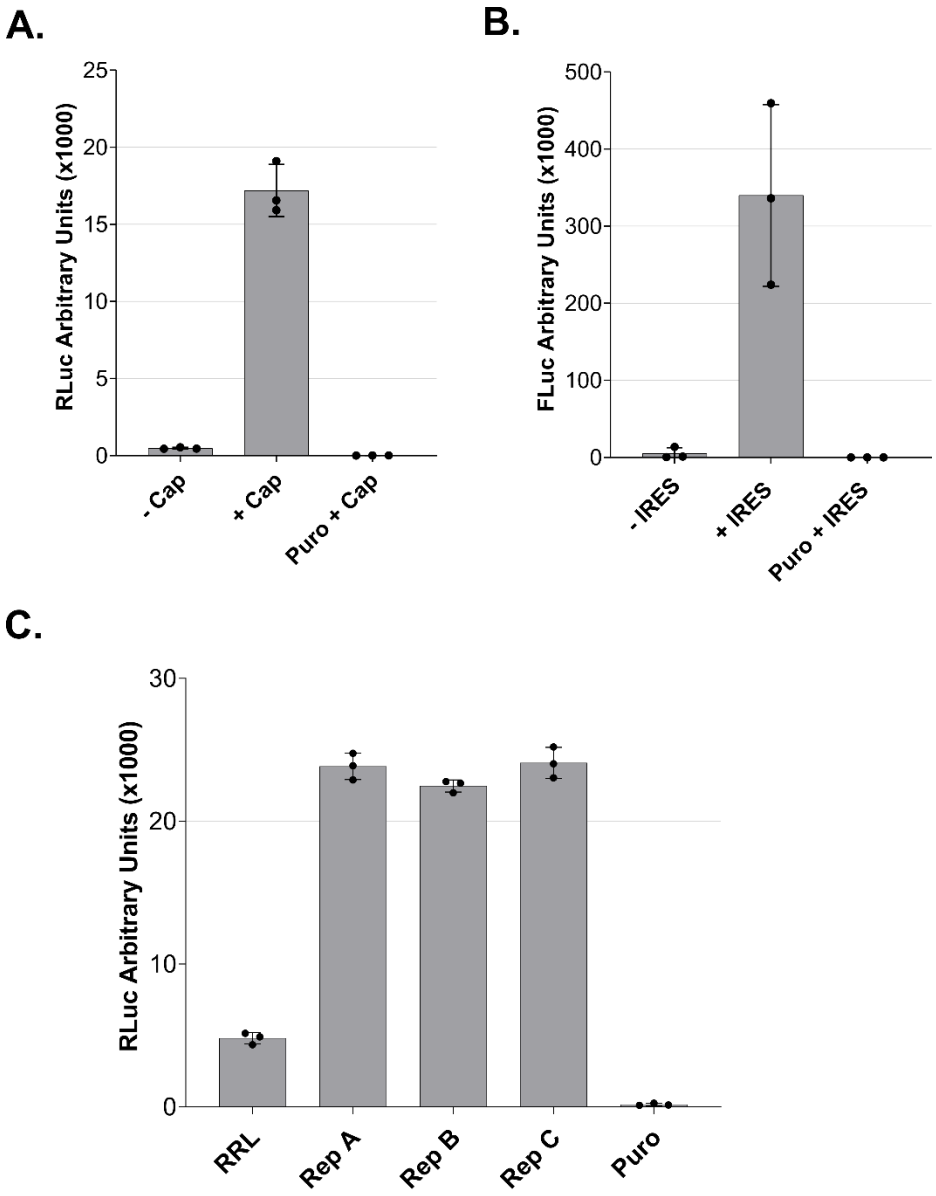

Figure 4: A. Comparison RLuc measurements of *in vitro* translation reactions of dual centrifugation-treated lysates with uncapped (-Cap) or capped (+Cap) mRNAs. **B.** Comparison of Firefly Luc measurements of *in vitro* translation reactions of DC-treated lysates using dual-luciferase reporters with or without an ECMV-IRES upstream of the Firefly ORF. **C.** *In vitro* translation using Rabbit reticulocyte lysate (RRL) and three HeLa S3 lysates. Rluc activity measurements are depicted as arbitrary units (AU) of luminescence. The dots depict the values of three individual experiments (biological replicates) that were measured each three times (technical replicates).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Silverman AD, Karim AS, Jewett MC. Cell-free gene expression: an expanded repertoire of applications. Nat Rev Genet. 2020 Mar 28;21(3):151–70.

2. Hammerling MJ, Krüger A, Jewett MC. Strategies for in vitro engineering of the translation machinery. Nucleic Acids Res. 2020 Feb 20;48(3):1068–83.

3. Nirenberg M, Leder P, Bernfield M, Brimacombe R, Trupin J, Rottman F, et al. RNA codewords and protein synthesis, VII. On the general nature of the RNA code. Proc Natl Acad Sci U S A. 1965;53(5):1161–8.

4. Nirenberg MW, Matthaei JH. The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribonucleotides. Proc Natl Acad Sci. 1961 Oct 1;47(10):1588–602.

5. Martin RW, Des Soye BJ, Kwon Y-C, Kay J, Davis RG, Thomas PM, et al. Cell-free protein synthesis from genomically recoded bacteria enables multisite incorporation of noncanonical amino acids. Nat Commun. 2018 Dec 23;9(1):1203.

6. Aleksashin NA, Szal T, D'Aquino AE, Jewett MC, Vázquez-Laslop N, Mankin AS. A fully orthogonal system for protein synthesis in bacterial cells. Nat Commun. 2020;11(1):1–11.

7. Zemella A, Thoring L, Hoffmeister C, Kubick S. Cell-Free Protein Synthesis: Pros and Cons of Prokaryotic and Eukaryotic Systems. ChemBioChem. 2015;16(17):2420–31.

8. Pelham HRB, Jackson RJ. An Efficient mRNA-Dependent Translation System from Reticulocyte Lysates. Eur J Biochem. 1976 Aug 1;67(1):247–56.

9. Pisareva VP, Muslimov IA, Tcherepanov A, Pisarev A V. Characterization of Novel Ribosome-Associated Endoribonuclease SLFN14 from Rabbit Reticulocytes. Biochemistry. 2015 Jun 2;54(21):3286–301.

10. Carlson ED, Gan R, Hodgman CE, Jewett MC. Cell-free protein synthesis: Applications come of age. Vol. 30, Biotechnology Advances. Elsevier; 2012. p. 1185–94.

11. Brödel AK, Sonnabend A, Kubick S. Cell-free protein expression based on extracts from CHO cells. Biotechnol Bioeng. 2014 Jan 6;111(1):25–36.

12. Bergamini G, Preiss T, Hentze MW. Picornavirus IRESes and the poly(A) tail jointly promote cap-independent translation in a mammalian cell-free system. RNA. 2000 Dec;6(12):S1355838200001679.

13. Mikami S, Masutani M, Sonenberg N, Yokoyama S, Imataka H. An efficient mammalian cell-free translation system supplemented with translation factors. Protein Expr Purif. 2006 Apr 1;46(2):348–57.

14. Rakotondrafara AM, Hentze MW. An efficient factor-depleted mammalian in vitro translation system. Nat Protoc. 2011 May 7;6(5):563–71.

15. Karousis ED, Gurzeler L-A, Annibaldis G, Dreos R, Mühlemann O. Human NMD ensues independently of stable ribosome stalling. Nat Commun. 2020 Dec 17;11(1):4134.

16. Hagedorn M, Bögershausen A, Rischer M, Schubert R, Massing U. Dual centrifugation – A new technique for nanomilling of poorly soluble drugs and formulation screening by an DoE-approach. Int J Pharm. 2017 Sep 15;530(1–2):79–88.

17. Massing U, Ingebrigtsen SG, Škalko-Basnet N, Holsæter AM. Dual Centrifugation - A Novel “in-vial” Liposome Processing Technique. In: Liposomes. InTech; 2017.

18. Schubert K, Karousis ED, Jomaa A, Scaiola A, Echeverria B, Gurzeler L-A, et al. SARS-CoV-2 Nsp1 binds the ribosomal mRNA channel to inhibit translation. Nat

Struct Mol Biol. 2020 Sep 9;1–8.

19. Grentzmann G, Ingram JA, Kelly PJ, Gesteland RF, Atkins JF. A dual-luciferase reporter system for studying recoding signals. RNA. 1998 Apr;4(4):479–86.

20. Pelletier J, Sonenberg N. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature. 1988 Jul 1;334(6180):320–5.

21. Jang SK, Kräusslich HG, Nicklin MJ, Duke GM, Palmenberg AC, 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(8):2636–43.