



Protocols

From gibbon assembly to cell-free expression: A guide to recombinant plasmid and toscana virus N protein production

Laura Dionisi^{a,*}, Alessandra Mistral De Pascali^a, Ludovica Ingletto^a, Giada Forlani^b, Gianni Gori Savellini^c, Massimiliano Guerra^d, Martina Brandolini^d, Claudia Colosimo^a, Giulia Gatti^d, Willian Cardoso Ferreira^a, Laura Grumiro^d, Giorgio Dirani^d, Silvia Zannoli^d, Maria Grazia Cusi^c, Monica Cricca^{a,d}, Alessandra Scagliarini^a, Vittorio Sambri^{a,d}

^a University of Bologna, Department of Medical and Surgical Sciences, Bologna, Italy

^b University of Freiburg, Institute of Biology II, Freiburg, Germany

^c University of Siena, Department of Medical Biotechnologies, Siena, Italy

^d Greater Romagna Hub, Microbiology Unit, Cesena, Italy

ARTICLE INFO

Keywords:

Toscana Virus

Recombinant Protein

Gibson Cloning

In-Vitro Protein Synthesis

ABSTRACT

Toscana virus (TOSV) is a mosquito-borne phlebovirus endemic to the Mediterranean region and an important cause of arboviral infections in humans, particularly during the summer months. Although TOSV infections are often asymptomatic or mild, the virus can cause neuroinvasive disease, including meningitis and encephalitis, highlighting the need for rapid and reliable tools for its detection and characterization. N protein was identified as a promising diagnostic target for TOSV due to its accumulation in infected cells and its role as primary immune target. The aim of this study was to establish a rapid and easily adaptable workflow for the recombinant production of the nucleoprotein (N) of Toscana virus (TOSV). We introduced a Gibson® Assembly-based cloning strategy using the pT7CFE1-CHis plasmid, specifically selected for its compatibility with cell free protein expression systems. The Gibson® approach enabled fast and efficient construct generation, overcoming the constraints typically associated with classical restriction-based cloning. Subsequently, cell free in vitro synthesis produced the recombinant protein within 16 h, enabling an efficient production. The yield was sufficient to perform downstream assays, including Western blot, thereby demonstrating the suitability of this combined strategy for rapid antigen prototyping and preliminary characterization. Our results highlight the recombinant TOSV antigen for diagnostics to support timely and disease detection and control

1. Introduction

The Toscana virus (*Phlebovirus toscanaense*) belongs to the *Phlebovirus* genus in the *Phenuiviridae* family, *Bunyavirales* order (Sasaya et al., 2023). Phleboviruses are enveloped, spherical viruses. The genome is a negative-sense, single-stranded RNA. The three RNA segments designated as large (L), medium (M), and small (S) encode the RNA-dependent RNA polymerase, the envelope glycoproteins, and the nucleoprotein (N), respectively (Moriconi et al., 2017). *Phlebovirus* genus comprises 10 species and more than 100 antigenically different viruses divided into two groups: those transmitted by sandflies and those transmitted by ticks (García San Miguel et al., 2021). Sandfly fever viruses (SFV) comprise three serotypes: Sicilian (SFVS), Naples (SFVN),

and Toscana (TOSV) (Soldateschi et al., 1999). While SFVS and SFVN infection usually cause self-limited disease characterized by fever, myalgia, and headache (Soldateschi et al., 1999) or asymptomatic (García San Miguel et al., 2021), TOSV infection is of particular clinical relevance since it is known to cause central nervous system (CNS) infection, leading to meningitis and meningoencephalitis (García San Miguel et al., 2021; Soldateschi et al., 1999). However, the high seroprevalence in endemic populations against the relative infrequency of disease implies that most infections are asymptomatic or subclinical: seroprevalence in endemic regions spans roughly 6–25% depending on geographic location, age, and exposure, yet only a small minority of those infected develop overt disease (Emeto et al., 2026). Among those who become unwell, a proportion has only a brief, self-limited febrile

* Corresponding author.

E-mail address: dionisi.laura@libero.it (L. Dionisi).

<https://doi.org/10.1016/j.jviromet.2026.115455>

Received 27 May 2026; Received in revised form 29 August 2026; Accepted 31 August 2026

Available online 2 September 2026

0166-0934/© 2026 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

illness with fever, malaise, myalgia, and arthralgia lasting a few days and without headache, meningism, or altered mentation (Emeto et al., 2026).

Genetic studies have identified the existence of three lineages of TOSV (A, B, and C), which do not differ in virulence or clinical manifestations (Toscana Virus Infection, ECDC, 2023). Specific geographic areas are associated with the circulation of each lineage (Toscana Virus Infection, ECDC, 2023). For instance, lineage A includes viral strains circulating in Italy (Ayhan & Charrel, 2020).

More than 250 million people in the Mediterranean region may be exposed to TOSV, with circulation confirmed in Italy, Türkiye, Greece, Corsica, and northern Africa (Sui et al., 2025). Given high travel numbers to the region, imported TOSV infection in non-endemic countries would require greater awareness and prompt diagnosis (Cusi & Savellini, 2011).

TOSV infection can be diagnosed by molecular detection of the virus or indirectly serological tests for specific antibodies (Ayhan & Charrel, 2020). There are only three commercial kits currently available for the serological diagnosis of TOSV: the Sandfly fever virus Mosaic 1 IIFA (EUROIMMUN), the recomLine Bunyavirus IgG/IgM immunoblot (Mikrogen), and the Enzywell TOSV IgG/IgM ELISA (DIESSE) (Ayhan & Charrel, 2020). This limited availability, together with the significant impact and spread of TOSV infection, highlight the need to expand the range of accessible diagnostic tests. Moreover, serology remains the cornerstone of Phleboviral infections diagnosis: since they are characterized by transient and low-level viremia, serological analysis is particularly valuable for TOSV infections diagnosis (Cusi & Savellini, 2011). Given the limited availability of standardized diagnostic assays and the need for more reliable tools to support surveillance of TOSV infection, recombinant viral proteins represent a valuable resource for improving assay performance and specificity, overcoming current diagnostic constraints. These approaches enable the generation of consistent, safe, and robust antigenic proteins to improve serological assay performance.

Among the viral antigens that could be used for this purpose, the TOSV nucleoprotein (N) appears promising due to its structural, functional, and immunological importance (Papageorgiou et al., 2024). The N protein is encoded by a sequence of 759 bp in the S segment of the negative viral ssRNA; the final product is a non-glycosylated 253-aminoacid protein, which associates with the RNA genome and, together with the polymerase, forms viral ribonucleoproteins (Överby et al., 2008). It is the most abundant viral protein in both infected cells and virions (Papageorgiou et al., 2024), and its marked accumulation in infected cells makes it the main antigen responsible for both IgM and IgG responses (Magurano & Nicoletti, 1999).

The use of N protein for diagnostic purposes is well-documented in the literature (Cusi & Savellini, 2011; Magurano & Nicoletti, 1999; Soldateschi et al., 1999), providing a strong rationale for its recombinant expression and use in serological tests. Analyses of N protein coding sequences from multiple TOSV strains isolated from patients and sandflies showed that the N protein is highly conserved, with at most a single aminoacid substitution among strains, suggesting that changes impairing its interaction with viral RNA are likely lethal and therefore not selected. Consequently, using the prototype strain as antigen should allow detection of anti-N antibodies even when the infecting strain differs (Magurano & Nicoletti, 1999), thereby making it a strong candidate for diagnostic use. Moreover, in TOSV-associated neurological disease, antibodies against linear N protein epitopes were consistently detected in both acute and convalescent sera, whereas responses to glycoproteins varied among patients (Di Bonito et al., 2002), confirming the immunodominance of the N protein. Analysis of aminoacid sequences of the *Phlebovirus* N proteins shows high identity, and at least five regions of the proteins may be considered highly conserved, leading to possible cross-reaction of the anti-N antibodies (Di Bonito et al., 2002). One study showed that specific TOSV antibodies cross-reacted with the N proteins of SFSV and, to a minor extent, with those of

SFNV, confirming that TOSV is antigenically more closely related to SFNV than to SFSV (Schwarz et al., 1996). In fact, the alignment of the aminoacid sequences of the N proteins of TOSV, SFNV, and SFSV – all members of the SFV group – revealed a sequence identity of approximately 89% between TOSV and SFNV, whereas the aminoacid sequence identity between TOSV and SFSV was only about 44%, further supporting previous findings about cross reactivity between those viral strains (Schwarz et al., 1996). The N protein remains a promising diagnostic target for TOSV due to its accumulation in infected cells and its role as primary immune target. Although some degree of cross-reactivity with N-protein of other Phleboviruses is possible and documented (Di Bonito et al., 2002; Schwarz et al., 1996), TOSV is the only member of this group commonly associated with neuroinvasive disease, making seropositivity in neurological cases highly indicative of TOSV infection.

At the core of all serological techniques lie recombinant proteins. They are generated through recombinant DNA technologies, which enable the insertion of a protein encoding gene of interest into a host organism (such as bacteria, yeasts, animal, or plant cells) enabling controlled and reproducible expression of the target antigen (Schütz et al., 2023). An alternative and easy to use approach is the cell-free protein synthesis (CFPS), a platform emerged as a strategy to prototype expression or to produce proteins that are toxic or poorly expressed in cellular systems (Sword et al., 2024). CFPS enables high-throughput protein screening, is easily adaptable and can synthesize proteins within hours. Overall, the synthesized proteins could be used as reference material for the development of diagnostic assays, providing scalability, high-throughput screening compatibility and time efficiency (Woelbern & Ramm, 2024).

Similarly, the method used to obtain recombinant plasmid depends on the length and complexity of the gene (Bird et al., 2022), vectors, tags, and expression systems selected (Celie et al., 2016). In addition, laboratory habits, financial and instrumentation resources will influence the choice of a particular cloning strategy (Celie et al., 2016). Gene cloning, using restriction endonucleases and DNA ligases, has been employed successfully for over 40 years, but in the age of cheap DNA synthesis its limitations are becoming evident (Casini et al., 2015). Several innovative cloning techniques such as Gibson® Assembly (Gibson et al., 2009), PCR-based assembly, in vivo assembly – that exploits the natural DNA repair and recombination mechanisms present within a microorganism (Bird et al., 2022) – and TA cloning (Green & Sambrook, 2021), offer more flexible and efficient alternatives (Bird et al., 2022; Green & Sambrook, 2021).

We present a rapid, easy to use, and adaptable workflow for producing recombinant protein suitable for early evaluation of potential diagnostic antigen candidates, in which both cloning and expression strategies can be rapidly applied to a variety of constructs. In this work, we combined two complementary and innovative approaches: Gibson® Assembly was employed to efficiently clone the gene of interest into the pT7CFE1-Chis plasmid (Thermo Scientific™), a 3.5 kb vector optimized for CFPS with a C-terminal His₆ tag, and the cell-free protein synthesis strategy using the Thermo Scientific™ 1-Step Human High-Yield Mini IVT Kit to rapidly generate TOSV N recombinant antigen. A graphical representation of the entire workflow, including the duration of each processing step and cumulative working times, is presented in Fig. 1.

2. Materials and methods

2.1. Cell culture maintenance

Vero E6 (ATCC CRL 1585) cells obtained from the American Type Culture Collection and maintained under standard cell culture conditions (37 °C, 5% CO₂), in MEM (Euroclone) supplemented with 10% of heat inactivated foetal bovine serum (Fetal Bovine Serum South America origin EU Approved- Highly Tested, EuroClone), 1% glutamine (Glutamine 100X 200 mM, EuroClone), and 1% Penicillin/Streptomycin (Penicillin-Streptomycin solution 100X EuroClone). Cells were splitted

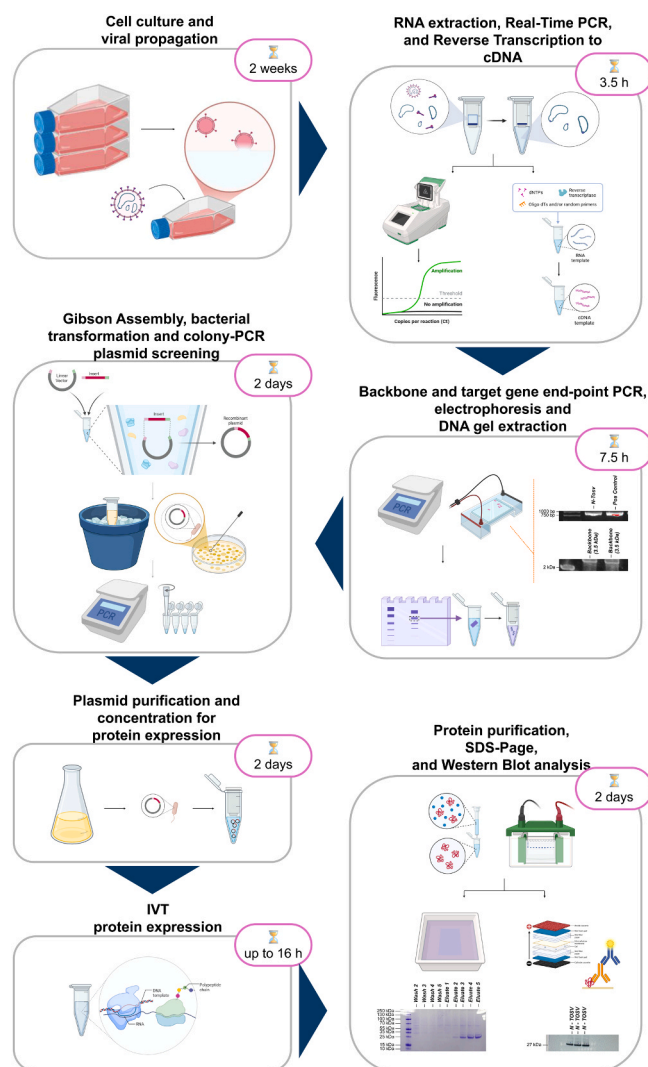


Fig. 1. Schematic representation of the workflow. Comprehensive workflow of the experimental protocol, showing the ordered progression of preparatory, processing, and analytical steps. Each box represents a specific phase of the protocol, which is described in detail in the following sections, and includes the approximate time required for its execution. Image created with BioRender.

1–2 times per week with a 1:3 or 1:4 dilution.

2.2. Viral propagation and infection kinetics monitoring

The TOSV strain RN_050724 (passage 5), used for viral propagation, was originally isolated from the cerebrospinal fluid of a confirmed positive patient (Brandolini et al., 2025). Its genome has been deposited and classified as lineage A. The viral inoculum was prepared based on its titer (1×10^7 TCID₅₀) and the target 0.5 multiplicity of infection (MOI) was calculated using the formula below.

$$\frac{\text{TCID} \times 0.7}{\text{number of cells to be infected}} = \text{MOI}$$

Vero E6 cells were seeded in T25 flasks and, upon reaching 90% confluence ($\sim 2.5 \times 10^6$ cells), the culture medium was removed. Cells were washed with 5 mL of phosphate-buffered saline (PBS), and viral inoculum was diluted to a final volume of 1 mL in DMEM with 2% of FBS (Desmyter et al., 1968). Viral adsorption proceeded for 3 h at 37°C, after which 7 mL of DMEM with 2% of FBS were added. Infection was monitored daily by assessing cytopathic effect (CPE) using a light microscope at 20X magnification, and supernatant aliquots were taken

each 24 h for Real-Time PCR (RT-PCR) to evaluate infection kinetics. AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems™) were used to perform the PCR. The amplification was carried out in 20 µL reaction, as described in Table 1. For RT-PCR the primers and probes (Table 2) developed by Thirion et al. 2021 were employed at the suggested concentrations. The PCR conditions selected for amplification were previously described by Brandolini et al., 2025. Cell culture supernatants were harvested at 72–96 h post-infection corresponding to RT-PCR Ct values of 9–12.

2.3. Viral RNA extraction

The infected culture medium was clarified by centrifugation for 5 min at 300xg and 200 µL were used for viral RNA extraction with RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol.

2.4. Retrotranscription to cDNA

For reverse transcription with SuperScript™ III Reverse Transcriptase (Invitrogen™), 7 µL of RNA were used per reaction to generate cDNA, following the manufacturer's protocol.

2.5. Designing of forward and reverse primer to perform the Gibson® Assembly

Forward and reverse primers were designed to amplify both the insert and the backbone, to generate terminal overlapping regions (typically 20–40 bp) between the insert (N gene of TOSV) and the backbone (pT7CFE1-CHis plasmid, Thermo Scientific™), enabling seamless assembly through the Gibson® Assembly method. The primer designs for N gene amplification were based on a reference sequence from deposited TOSV Lineage A strain (KM275773.1). This is the only lineage currently circulating in Italy (Ayhan & Charrel, 2020). For this purpose, free online tool Benchling (Biology Software Benchling. San Francisco CA, 2025) was used. The resulting primer set is shown in Table 3.

2.6. End-Point PCR of nucleoprotein gene and of pT7CFE1-CHis backbone

TOSV cDNA was used as a template to amplify the N gene. PCR amplification conditions selected for amplification were 94°C for 2 min, (95°C for 30 s, 56°C for 30 s, 72°C for 45 s) for 35 cycles, and a final elongation step of 72°C for 7 min with the given primer set. The amplification was carried out in 25 µL reaction using FastStart High Fidelity (Roche), with 6% DMSO (final concentration).

The pT7CFE1-CHis (10 ng) (Thermo Scientific™) vector was used as a template to obtain the linear backbone. The PCR amplification conditions selected for amplification were 94°C for 2 min, (94°C for 30 s, 60°C for 30 s, 68°C for 5 min) for 10 cycles, (94°C for 30 s, 60°C for 30 s, 68°C for 5 min and 20 s) for 25 cycles, and a final elongation step of 72°C for

Table 1

Composition of the 20 µL RT-PCR reaction mix described by Thirion et al., 2021.

Component	Volume	Final Concentration
Enzyme	5 µL	/
STOS-F	2 µL	0.8 mM
STOS-R	2 µL	0.8 mM
STOS-P	0.4 µL	0.16 mM
TOS-F	1 µL	0.4 mM
TOS-R	1 µL	0.4 mM
TOS-P	0.4 µL	0.16 mM
TOS-IMT-F	0.8 µL	0.32 mM
TOS-IMT-R	0.8 µL	0.32 mM
TOS-IMT-P	0.4 µL	0.16 mM
DNTPs mix (10 mM each)	2 µL	0.8 mM (each)
Template	5 µL	/

Table 2

Primer sequences (5'→3') used for RT-PCR detection of TOSV as described by Thirion et al., 2021.

STOS-F	TGCTTTTCTTGATGAGTCTGCAG
STOS-R	CAATGCGCTTYGGRTCAA
STOS-P	FAM-ATCAATGCATGGGTRAATGAGTTTGCTTACC-TAMRA
TOS-F	GGGTGCATCATGGCTCTT
TOS-R	GCAGRGACACCATCACTCTGTC
TOS-P	FAM-CAATGGCATCCATAGTGGTCCCAGA-TAMRA
TOS-IMT-F	TCTCCCAGGAAATGACATCC
TOS-IMT-R	AGATGGGWTCTCTGGTCAT
TOS-IMT-P	FAM-TGTGGTYCAAGCAGCACGGGTG-TAMRA

Table 3

Primers used for amplifying the backbone pT7CFE1-Chis and the nucleocapsid gene from viral cDNA.

Backbone forward	ATCGCGCTAAGTTGGCAAGCACCACCACCACCACTG
Backbone reverse	TCCGCGATAATCTCTGCTGACATATGGGTGGTGGCCATATTATC
Gene N forward	ATATGGCCACCACCATATGTCAGACGAGAATTATCGCGA
Gene N reverse	CAGTGGTGGTGGTGGTGGTCTTGCCAACTTAGCGCGAT

7 min for each primer set. The amplification was carried out in 25 µL reaction using FastStart High Fidelity (Roche), with 6% DMSO (final concentration).

2.7. DNA gel purification

Before proceeding to the cloning step, the DNA was gel purified using the Monarch® Spin DNA Gel Extraction Kit (NewEngland Biolabs). For the purification of the target amplicon, gels with reduced thickness and low agarose concentrations (0.5–1%) were casted, to increase the final yield of purified DNA. After electrophoresis the gel was longitudinally cut, as shown in Fig. 2. Only the molecular weight marker lane and the first PCR product lane were post-run stained (15 min in 1X TBE with SYBR® Safe, per Thermo Scientific™) and exposed to UV light to excise the band of interest. The stained and unstained gel sections were then realigned, using the stained portion as a guide to excise the corresponding regions from the remaining agarose gel. Multiple gel slices were placed into the microcentrifuge tube and purified according to manufacturer's protocol.

2.8. Gibson® assembly

The N gene and the linearized backbone, both purified from the agarose gel, were quantified to calculate the substrate volumes to include in the cloning reaction. The NEBioCalculator tool (New England Biolabs, Ipswich, MA, USA) was used to calculate the appropriate volume of reagents (µL). The backbone was set to 50 ng and based on the insert size and desired molar ratio, the tool provided the quantity of

insert in the reaction to obtain a 3:1 DNA-to-plasmid ratio. The 10 µL cloning reaction was incubated for 15 min at 50°C (In-Fusion® HD Cloning Kit User Manual, Takara Bio).

2.9. Bacterial transformation

For the transformation, 50 µL of competent *E. coli* DH5α Cells (Thermo Scientific™) were transformed with the Gibson® Cloning reaction (10 µL). In parallel, a control Gibson® reaction (backbone omitted) bacterial transformation was performed. Cells were incubated on ice for 30 min and then given a heat shock at 42°C for 45 s. S.O.C Medium (350 µL) (Invitrogen™) was added to cells before incubating for 1 h at 37°C at 250 rpm shaking. Transformed cells (200 µL) were plated onto selective Luria Broth (Invitrogen™) agar plates supplemented with 100 µg/mL ampicillin (Gibco™), since the expression vector carried the ampicillin resistance gene, enabling selection of successfully transformed cells. Plates were incubated overnight at 37°C and single colonies were subsequently streaked onto fresh LB agar plate supplemented with ampicillin (100 µg/mL) to avoid false-positive PCR results due to residual free-DNA on the plate.

2.10. Colony PCR

The End-Point colony PCR was performed using Taq DNA Polymerase (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. PCR amplification condition selected for amplification was 95°C for 15 min, (94°C for 1 min, 62°C for 1 min, 72°C for 1 min) for total of 30 cycles, and a final elongation step of 72°C for 10 min. Primers were designed to amplify the recombinant vector: the forward primer anneals to a vector-encoded region upstream of the insertion site, while the reverse primer targets the 3' end of the N gene. The forward primer sequence was [5'-ACCCCAT TGTATGGGATCTGATC-3'], paired with the Gene N reverse primer listed in Table 3, used to a final concentration of 200 µM. The amplification was carried out in 25 µL reaction, where the colonies were directly resuspended.

2.11. Starter culture for mini-prep plasmid extraction

Plasmid amplifications and extractions were performed as described by GeneJET™ Plasmid Miniprep's protocol (Thermo Scientific™). One single positive colony was inoculated into 5 mL of LB broth supplemented with ampicillin (100 µg/mL) and incubated overnight at 37°C with agitation at 250 rpm. Upon completion of the extraction, the plasmid was sequenced with Nanopore, to exclude the presence of mutations within regulatory regions.

2.12. Oxford nanopore sequencing

The starting material and sequencing libraries were quantified using a Qubit 2.0 Fluorometer (Invitrogen), according to the manufacturer's instructions. Sequencing was performed by an external service provider

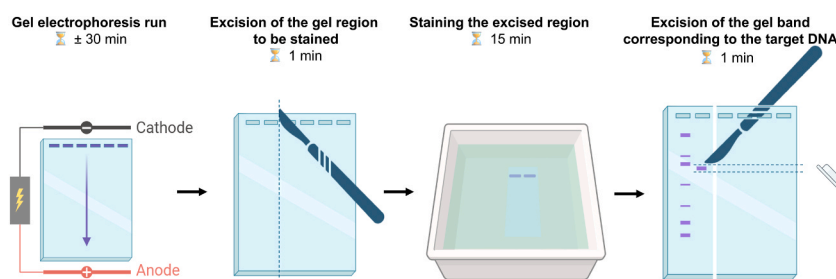


Fig. 2. Post-run gel staining and band excision. The portion of the gel containing the molecular weight marker lanes and one lane with the PCR product was first isolated and exposed to UV light to excise the band of interest. The stained and unstained gel sections were then realigned, using the stained portion as a guide to excise the corresponding regions from the remaining agarose gel (Image created with BioRender).

using an Oxford Nanopore MinION platform equipped with a FLO-MIN114 flow cell (Flow Cell ID: FBE31492). Libraries were prepared using the SQK-NBD114–96 kit and sequenced for up to 72 h. Basecalling was performed using MinKNOW v25.09.18 and Dorado v7.11.2.

2.13. Starter culture for Maxi-Prep plasmid extraction

Upon confirmation of the integrity of the plasmid regulatory sequences, plasmid amplification and extraction were performed as described by GeneJET™ Plasmid Maxiprep's protocol (Thermo Scientific™). The colony containing the candidate plasmid was inoculated into 5 mL of LB broth supplemented with ampicillin (100 µg/mL) and incubated for 8 h/37°C with shaking at 250 rpm. The resulting starter culture was diluted 1:1000 in fresh LB broth containing ampicillin and grown overnight under the same conditions. The following day, cells were harvested when the culture reached an OD of 2–3.

2.14. DNA clean-up

In this experimental set up we decided to purify 30 µL and 50 µL of plasmid DNA. Two volumes of 100% ethanol and 1/10 vol of 3 M sodium acetate were added to plasmid DNA and incubated at -20°C for 15 min. The mixture was then centrifuged for 15 min at 14000 x g, and the supernatant was removed without disturbing the small visible pellet. The pellet was washed with 70% ethanol and centrifuged for 5 min at 14000 x g. After removal of the supernatant, the pellet was air-dried under laminar flow for 5 min, resuspended in half of the initial volume of nuclease-free water, and quantified using a NanoDrop spectrophotometer.

2.15. In-vitro protein synthesis with 1-Step human high-yield Mini IVT kit

The reaction was prepared according to the protocol provided with the 1-Step Human High-Yield Mini IVT Kit (Thermo Scientific™), using 4000 ng of recombinant plasmid. In parallel, a GFP control reaction was included, to verify protein expression activity. Both reactions were incubated at 30 °C for 16 h with shaking at 900 rpm and subsequently stored at -20 °C until further use.

2.16. Protein purification

Protein purification was performed using HisPur™ Ni-NTA Spin Columns (Thermo Scientific™) according to the manufacturer's instructions. The imidazole concentrations used in the equilibration, wash, and elution buffers were adjusted to refine resin binding, minimize nonspecific washout, and maximize elution efficiency. Specifically, the Equilibration Buffer was supplemented with 25 mM imidazole, the Wash Buffer with 20 mM imidazole, and the Elution Buffer with 500 mM imidazole. The pH of all solutions was maintained between 7.0 and 8.0. Five washing steps and up to eight elution steps were carried out with the previously prepared Wash and Elution Buffers.

2.17. SDS-PAGE and Coomassie Gel staining

Aliquots of wash fractions and purified protein (16.5 µL each) were mixed with 6.25 µL of NuPAGE LDS Sample Buffer (4 ×) (Thermo Scientific™) and 2.5 µL of NuPAGE Reducing Agent (4 ×) (Thermo Scientific™). Samples were heated at 70 °C for 10 min prior to loading onto NuPAGE® Novex® 4–12% Bis-Tris Gels (Thermo Scientific™). Electrophoresis was performed according to the manufacturer's instructions. Gels were then incubated with 20 mL of Imperial™ Protein Stain (Thermo Scientific™) for 2 h at room temperature under gentle agitation. Subsequently, the staining solution was replaced with 100 mL of ultrapure water, and gels were incubated overnight at room temperature with gentle shaking to reduce background staining. The three fractions that showed visible protein bands on the Coomassie-stained SDS-PAGE

gel, were quantified with NanoDrop spectrophotometer.

2.18. Western blot

For Western blot analysis, 2.48 µg of protein previously loaded into a NuPAGE Novex® 4–12% Bis-Tris Gels (Thermo Scientific™) polyacrylamide gel was transferred onto the nitrocellulose membrane using Power Blotter Select Transfer Stacks (Invitrogen™) and the Power Blotter System (Invitrogen™) following option A (1 mini-gel; 1.3 A). The membrane was then blocked for 1 h at room temperature with 15 mL of StartingBlock™ Blocking Buffer (Thermo Scientific™) supplemented with 0.05% Tween™20 (Sigma-Aldrich) under gentle shaking. Primary antibody incubation was performed using a mouse IgG antibody against protein N (Ref: 022A-05254, EVA-G), diluted 1:50 in a solution consisting of 2 mL StartingBlock™ Blocking Buffer (Thermo Scientific™), and 18 mL PBS (Euroclone) containing 0.05% Tween™20. The membrane was incubated overnight at 4 °C with gentle agitation.

Following incubation, the membrane was washed three times for 10 min each with PBS containing 0.05% Tween™20. The secondary antibody, Goat anti-Mouse IgG (H+L) HRP (Invitrogen™), was diluted 1:5000 in 2 mL StartingBlock™ Blocking Buffer and 18 mL PBS with 0.05% Tween™20 and incubated for 1 h at room temperature with gentle agitation. The membrane was then washed six times for 5 min each in PBS with 0.05% Tween™20. Chemiluminescent detection was performed using Pierce™ ECL Western Blotting Substrate (Invitrogen™), prepared by mixing equal volumes of the two components. After 5 min incubation at room temperature, signals were acquired using the iBright™ Imaging System (Invitrogen™) with SmartRange settings.

3. Results

3.1. Gibson assembly and upstream preparations

The protocol for synthesis of the pT7CFE1-CHis vector recombined with the N gene sequence of TOSV was successfully carried out through PCR amplification of both substrates. The PCR required only little optimization; however, gel purification of both the plasmid and the gene after amplification was necessary. Strategies to maximize plasmid and target gene recovery yields were implemented, by processing multiple bands on a single column and using very thin cast agarose gels (as reported in Materials and Methods). This allowed us to obtain higher concentrations for both the plasmid (>35 ng/µL) and the N gene (>20 ng/µL).

3.2. Screening results of transformed E. coli DH5a and plasmid extraction quality

Bacterial transformation yielded 58 colonies on the selective agar plate. The control Gibson® ligation reaction (backbone omitted) typically yielded no colonies or only a few (≈2–3). 30 colonies (51.7%) were picked for colony PCR, and 10 of them (33%) were positive by colony-PCR screening. Several PCR-positive colonies were grown overnight (≈16 h) and plasmids extracted with the GeneJET Plasmid MiniPrep Kit (Thermo Scientific™), yielded excellent DNA concentrations and A_{260/280} and A_{260/230} ratios results (Table 4). A single sequenced colony was then scaled up using GeneJET Plasmid MaxiPrep Kit (Thermo Scientific™); yields and extraction results are shown in Table 5.

3.3. Oxford nanopore sequencing results

Quality assessment of the FASTQ files was performed using FastQC v0.12.1. A total of 15,357 reads were generated, corresponding to 65,148,751 sequenced bases, with an average read length of 4242.3 bp. Comparison of the sequencing results with the corresponding reference sequences confirmed the absence of mutations or deletions in critical

Table 4

Expected yields and DNA quality after Mini prep plasmid extraction. This table reports the results of six different extractions obtained from six 5 mL bacterial cultures.

Concentration (ng/uL)	A260/280	A260/230
212.8 (SD = 16.7; n = 6)	1.90	2.24
192.5 (SD = 16.7; n = 6)	1.89	2.17
191.4 (SD = 16.7; n = 6)	1.89	2.13
186.5 (SD = 16.7; n = 6)	1.90	2.20
228.8 (SD = 16.7; n = 6)	1.90	2.25
215.1 (SD = 16.7; n = 6)	1.89	2.22

Table 5

Expected yields and DNA quality after Maxi prep plasmid extraction. This table reports the results of six different extractions obtained from six 250 mL bacterial cultures.

Concentration (ng/uL)	A260/280	A260/230
459.1 (SD = 52.20; n = 6)	1.91	2.29
456.7 (SD = 52.20; n = 6)	1.91	2.30
385.2 (SD = 52.20; n = 6)	1.91	2.29
459.1 (SD = 52.20; n = 6)	1.91	2.29
337.2 (SD = 52.20; n = 6)	1.90	2.28
456.7 (SD = 52.20; n = 6)	1.91	2.30

regulatory elements.

3.4. DNA clean up, in vitro protein synthesis, purification and Western Blot Analysis

The DNA clean up procedure yielded excellent results, as reported in the Table 6. After protein production with the Thermo Scientific™ 1-Step Human High-Yield Mini IVT Kit, the HisPur™ Ni-NTA Spin Columns (Thermo Scientific™) allowed the purification of the target protein. Both the SDS-Page analysis and the Western blot yielded satisfactory results, confirming both the presence and immunogenicity of the N recombinant protein. Results of SDS-PAGE gel staining and Western Blot are shown in Figs. 3A and 3B.

The three fractions that showed visible protein bands on the Coomassie-stained SDS-PAGE gel, corresponding to elution fractions 3, 4, and 5, were quantified and showed protein concentrations of 60, 111, and 150 µg/mL, respectively. Based on the loaded volume (16.5 µL), the corresponding amounts of protein loaded were 0.99, 1.83, and 2.48 µg, respectively. The same volume of Elution fraction 5 was used for the Western blot, corresponding to 2.48 µg of protein per well.

4. Discussion

The main focus of this study was to develop an efficient, optimized protocol to produce the TOSV N protein of TOSV for serological diagnosis, as it is the major immunogen during acute TOSV infection (Cusi & Savellini, 2011). We evaluated protein production using the pT7CFE1-Chis vector a 1-Step Human High-Yield Mini IVT Kit (Thermo

Table 6

Expected yields of purity and concentration after DNA clean-up protocol.

Initial volume subjected to the protocol	Concentration (ng/uL)	A260/280	A260/230
30 uL	682.8 (SD = 21.2; n = 2)	1.87	2.28
30 uL	652.8 (SD = 21.2; n = 2)	1.82	2.27
50 uL	903.1 (SD = 166.1; n = 2)	1.89	2.26
50 uL	1138 (SD = 166.1; n = 2)	1.87	2.25

Scientific™) – a cell-free system optimized for this vector. The target gene was efficiently cloned into the pT7CFE1-Chis vector with Gibson® Assembly.

Given the growing number of alternatives to classical restriction enzyme-based cloning and the wide range of expression systems now available, it is essential to examine these options for molecular cloning and protein synthesis (Celie et al., 2016). To date, there are few fully integrated end-to-end protocol that describes the entire workflow from viral propagation to recombinant antigen validation within a single methodological framework. Because the available information is scattered across numerous independent studies performed under varying conditions, researchers must piece together data from diverse sources. This makes the implementation of the complete procedure particularly challenging, especially for laboratories approaching recombinant viral protein production for the first time.

Expanding methodological knowledge and promoting research on TOSV is important for development of diagnostic tools, enhance patients' management and epidemiological surveillance. The access to detailed, optimized, and validated protocols for the identification and production of potential diagnostic targets may facilitate research not only on TOSV but also in other neglected infectious diseases. Overall, several phases of this methodology can be easily adapted to clone different pathogen sequences, enabling the production and characterization of a wide range of recombinant proteins and diagnostic targets, thus increasing the versatility and applicability of the protocol across different areas of virology, diagnostics, and biotechnology research.

Optimization of each individual step of the protocol produced overall outcomes. The first critical parameter is the quantity of DNA substrates used for Gibson® Assembly. We therefore optimized the agarose-gel purification workflow for both insert fragments and the backbone, increasing nucleic acid's concentration. We also performed a post-run staining since intercalating dyes change the charge and flexibility of DNA molecules and add weight, altering movement through the gel. The post-staining method is therefore the most accurate way to size DNA fragments, but it is more time consuming and expensive (Hall, 2020). Nonetheless, the staining solution can be reused multiple times without any detectable loss in performance if stored in the dark at room temperature. This practice helps to mitigate the overall cost associated with the use of larger quantities of intercalating dyes.

The broadening landscape of cloning methods and expression systems calls for a careful assessment of the available options for molecular cloning and protein synthesis. In our study, the Gibson® Assembly method proved highly advantageous, eliminating the need for restriction enzymes (Bird et al., 2022), which may be constrained by recognition sites within the target sequence and may require the use of enzymes with suboptimal activity (Casini et al., 2015). In some cases, inefficient digestion may also require changing the set of enzymes and adopting an alternative cloning strategy. By contrast, this assembly system requires only its master mix, the PCR amplified target gene product and subsequent purification to perform a successful directional cloning reaction. Moreover, compared with other rapid PCR-based cloning methods (such as TA-cloning provided by Thermo Scientific™), this approach can be applied to any available vector and ensures directional cloning, even for large or multiple inserts. Assembly reaction was performed with In-Fusion® HD (Takara Bio) enzyme mix, and the protocol did not require further optimization. The DNA-to-plasmid molar ratio used (3:1) proved to be successful; however, DNA:plasmid proportion can be increased for more challenging cloning reactions and in different experimental set ups.

Transformation of competent *E. coli DH5a* cells did not require any optimization. If needed, the operator can increase the number of visible colonies on selective plates by plating the entire volume of transformed cells. We emphasize the importance of including an additional colony-isolation step after transformation when colony PCR is planned. Restricting the colonies onto a clean plate helps prevent false-positive results caused by residual free DNA on the original transformation plate,

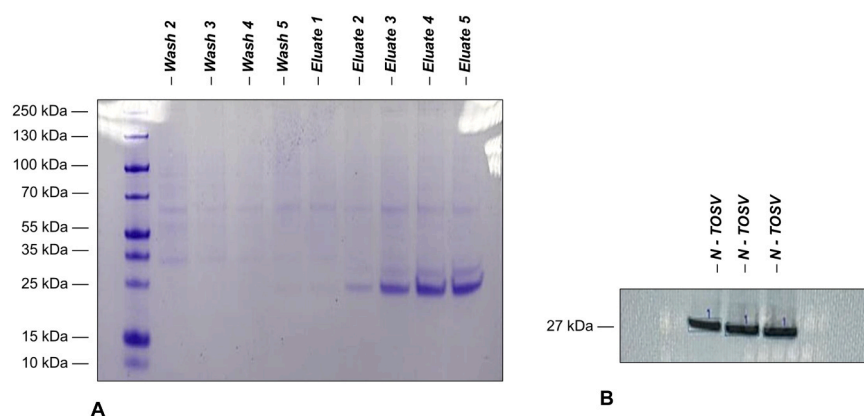


Fig. 3. Purification Profile and Immunodetection of N Protein. A. Coomassie-stained SDS-PAGE gel illustrating the protein purification profile, including the molecular weight marker, wash fractions (Wash 2 – Wash 5), and elution fractions (Elution 1 – Elution 5), in which the target protein is predominantly detected, allowing assessment of its apparent molecular weight and distribution across fractions. B. Western blot analysis of the N protein obtained from Elution 5, performed in triplicate to confirm the identity and reproducibility of detection of the target protein.

particularly if using primers that anneal exclusively within the insert sequence.

Plasmid amplifications and extractions were performed as described by Thermo Scientific™ GeneJET Plasmid Miniprep and Maxiprep, and both yielded optimal concentrations, $A_{260/280}$, and $A_{260/230}$ ratios, as shown in Tables 4 and 5. We carried out a 5 mL starter culture, as specified in the Miniprep protocol, to rapidly obtain sufficient plasmid for re-analysis and sequencing to verify the absence of possible mutations within critical coding or regulatory regions. Sequencing analysis confirmed the correct insertion of the selected sequence into the plasmid, with no mutations detected in relevant regions.

Before protein production, the recombinant plasmid was prepared with DNA clean up procedure. The volume chosen for the DNA clean-up was at the experimenter's discretion; in our case, an initial volume of 30 or 50 μ L was used. The final resuspension volume was set to half of the starting volume, thereby yielding a concentration of recombinant plasmid sufficient to meet the protein expression kit's requirement (4000 ng). For protein synthesis, the Thermo Scientific™ 1-Step Human High-Yield Mini IVT Kit – a cell-free protein expression system derived from HeLa cell extracts, which also supports proper glycosylation of the resulting protein products. This proved extremely simple to use and rapid (16 h for protein production), requiring small reaction volumes (100 μ L) that were nonetheless suitable for preliminary testing with Western blot after protein purification.

The recombinant N protein was purified using HisPur Ni-NTA Spin Columns (Thermo Scientific™), thanks to the C-terminal His₆ tag. In-house buffers were maintained at pH 7.1 – 8.0 to ensure histidine deprotonation and efficient nickel binding, as binding to the resin is compromised below pH 7 (Calinsky & Levy, 2024). The imidazole concentration was also increased to 20 mM in the equilibration buffer to prevent nonspecific binding of host-cell proteins to the resin. Finally, increasing the number of wash steps to five and performing up to six elutions maximized recovery of highly purified protein fractions. The purification result was not optimal, since some nonspecific proteins were still present in the elution fractions above the protein of interest (Fig. 3A). The target protein is clearly visible at approximately 25 kDa. Nevertheless, the overall result can be considered satisfactory, as the purification was performed using a kit requiring only a benchtop centrifuge without the need for additional purification techniques, such as size-exclusion chromatography (SEC).

Finally, the Western blot analysis of the recombinant N protein confirmed retention of antigenicity, indicating the antigen suitability for downstream functional and immunological assays for TOSV diagnosis.

One significant limitation of CFPS used for this protocol is the relatively low yield of the protein obtained, since this system can produce up

to 750 μ g/mL of protein in a single 100 μ L reaction volume. This method is therefore not competitive for large-scale production of protein samples, as conventional cell-based systems typically achieve much higher yields. Overall, the implementation of this protocol showed that it is feasible to efficiently produce reliable viral-specific recombinant protein in a time-saving manner and using standard laboratory equipment, thereby laying the groundwork for exploratory studies and future investigations. Nevertheless, the results obtained so far, as demonstrated by Western Blot analysis, suggest that this recombinant antigen represents a suitable tool for serological diagnosis.

CRediT authorship contribution statement

Monica Cricca: Validation, Supervision. **Giada Forlani:** Writing – review & editing, Visualization, Resources, Methodology. **Alessandra Scagliarini:** Writing – review & editing, Validation, Supervision, Resources. **Gianni Gori Savellini:** Writing – review & editing, Resources, Methodology. **Silvia Zannoli:** Writing – review & editing. **Alessandra Mistral De Pascali:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation. **Maria Grazia Cusi:** Writing – review & editing, Validation, Resources. **Ludovica Ingleto:** Writing – review & editing. **Claudia Colosimo:** Writing – review & editing. **Vittorio Sambri:** Validation, Supervision, Conceptualization. **Massimiliano Guerra:** Writing – review & editing. **Martina Brando-lini:** Writing – review & editing. **Laura Grumiro:** Writing – review & editing. **Giorgio Dirani:** Writing – review & editing. **Laura Dionisi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Giulia Gatti:** Writing – review & editing. **Willian Cardoso Ferreira:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank the members of the laboratory for their support. This work did not receive any specific funding.

Data availability

No data was used for the research described in the article.

References

- Ayhan, N., Charrel, R.N., 2020. An update on Toscana virus distribution, genetics, medical and diagnostic aspects. *Clin. Microbiol. Infect.* 26 (8), 1017–1023. <https://doi.org/10.1016/j.cmi.2019.12.015>.
- Benchling. San Francisco (CA): Benchling; 2025. Available from: <https://www.benchling.com/>.
- Bird, J.E., Marles-Wright, J., Giachino, A., 2022. A User's guide to golden gate cloning methods and standards. *ACS Synth. Biol.* 11 (11), 3551. <https://doi.org/10.1021/ACSSYNBIO.2C00355>.
- Brandolini, M., Ingletto, L., Dirani, G., Zannoli, S., Lapusneanu, A.M., Guerra, M., De Pascali, A.M., Lelli, D., Defilippo, F., Lavazza, A., Calzolari, M., Dottori, M., Dionisi, L., Colosimo, C., Gatti, G., Marzucco, A., Montanari, M.S., Grumiro, L., Cricca, M., Sambri, V., 2025. Development of a novel amplicon based whole-genome sequencing framework for improved surveillance of Toscana virus. *Commun. Biol.* 2025 81 8 (1), 714. <https://doi.org/10.1038/s42003-025-08159-9>.
- Calinsky, R., Levy, Y., 2024. Histidine in Proteins: pH-dependent interplay between π - π , Cation- π , and CH- π Interactions. *J. Chem. Theory Comput.* 20 (15), 6930. <https://doi.org/10.1021/acs.jctc.4c00606>.
- Casini, A., Storch, M., Baldwin, G.S., Ellis, T., 2015. Bricks and blueprints: methods and standards for DNA assembly. *Nat. Rev. Mol. Cell. Biol.* 16 (9), 568–576. <https://doi.org/10.1038/NRM4014>.
- Celie, P.H.N., Parret, A.H.A., Perrakis, A., 2016. Recombinant cloning strategies for protein expression. *Curr. Opin. Struct. Biol.* 38 (3), 145–154. <https://doi.org/10.1016/j.sbi.2016.06.010>.
- Cusi, M.G., Savellini, G.G., 2011. Diagnostic tools for Toscana virus infection. *Expert. Rev. Anti-Infect. Ther.* 9 (7), 799–805. <https://doi.org/10.1586/ERI.11.54>.
- Desmyter, J., Melnick, J.L., Rawls, W.E., 1968. Defectiveness of interferon production and of rubella virus interference in a line of african green monkey kidney cells (Vero). *J. Virol.* 2 (10), 955–961. <https://doi.org/10.1128/jvi.2.10.955-961.1968>.
- Di Bonito, P., Bosco, S., Mochi, S., Accardi, L., Ciufolini, M.G., Nicoletti, L., Giorgi, C., 2002. Human antibody response to Toscana virus glycoproteins expressed by recombinant baculovirus. *J. Med. Virol.* 68 (4), 615–619. <https://doi.org/10.1002/jmv.10227>.
- Emeto, T.I., Mittova, V., Makky, A., Elahi, P., & Awali, H. (2026). *Clinical review of Toscana virus: a Mediterranean Neuroinvasive Phlebovirus*. <https://doi.org/10.3389/fepid.2026.1909843>.
- European Centre for Disease Prevention and Control (ECDC). Toscana virus infection (2023). Retrieved 18 June 2026, from <https://www.ecdc.europa.eu/en/toscana-virus-infection>.
- García San Miguel, L., Sierra, M.J., Vazquez, A., Fernandez-Martínez, B., Molina, R., Sanchez-Seco, M.P., Lucientes, J., Figuerola, J., de Ory, F., Monge, S., Suarez, B., Simón, F., 2021. Phlebovirus-associated diseases transmitted by phlebotominae in Spain: Are we at risk? *Enferm. Infecc. Y. Microbiol. Clin. (Engl. Ed.)* 39 (7), 345–351. <https://doi.org/10.1016/j.eimce.2021.05.001>.
- Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., Smith, H.O., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* 2009 65 6 (5), 343–345. <https://doi.org/10.1038/nmeth.1318>.
- Green, M.R., Sambrook, J., 2021. Cloning polymerase chain reaction (pcr) products: Ta cloning. *Cold Spring Harb. Protoc.* 2021 (6), 242–246. <https://doi.org/10.1101/pdb.prot101303>.
- Hall, A.C., 2020. A comparison of DNA stains and staining methods for Agarose Gel Electrophoresis. *BioRxiv*, 568253. <https://doi.org/10.1101/568253>.
- Magurano, F., Nicoletti, L., 1999. Humoral response in toscana virus acute neurologic disease investigated by viral-protein-specific immunoassays. *Clin. Diagn. Lab. Immunol.* 6 (1), 55. <https://doi.org/10.1128/cdli.6.1.55-60.1999>.
- Moriconi, M., Rugna, G., Calzolari, M., Bellini, R., Albieri, A., Angelini, P., Cagarelli, R., Landini, M.P., Charrel, R.N., & Varani, S. (2017). *Phlebotomine sand fly-borne pathogens in the Mediterranean Basin: Human leishmaniasis and phlebovirus infections*. <https://doi.org/10.1371/journal.pntd.0005660>.
- Överby, A.K., Pettersson, R.F., Grünwald, K., Huiskonen, J.T., 2008. Insights into bunyavirus architecture from electron cryotomography of Uukuniemi virus. *Proc. Natl. Acad. Sci. USA* 105 (7), 2375–2379. <https://doi.org/10.1073/pnas.0708738105>.
- Papageorgiou, N., Baklouti, A., Lichiè, J., Desmyter, A., Canard, B., Coutard, B., Ferron, F., 2024. Structural flexibility of Toscana virus nucleoprotein in the presence of a single-chain camelid antibody. *Res. Pap. Acta Cryst.* 80, 113–122. <https://doi.org/10.1107/S2059798324000196>.
- Sasaya, T., Palacios, G., Briese, T., Serio, F.Di, Groschup, M.H., Neriya, Y., Song, J.W., Tomitaka, Y., 2023. ICTV virus taxonomy profile: Phenuiviridae 2023. *J. General. Virol.* 104 (9), 001893. <https://doi.org/10.1099/jgv.0.001893>.
- Schütz, A., Bernhard, F., Berrow, N., Buyel, J.F., Ferreira-da-Silva, F., Hausteraete, J., van den Heuvel, J., Hoffmann, J.E., de Marco, A., Peleg, Y., Suppmann, S., Unger, T., Vanhoucke, M., Witt, S., Remans, K., 2023. A concise guide to choosing suitable gene expression systems for recombinant protein production. *STAR Protoc.* 4 (4), 102572. <https://doi.org/10.1016/j.xpro.2023.102572>.
- Schwarz, T.F., Gilch, S., Pauli, C., Jäger, G., 1996. Immunoblot detection of antibodies to Toscana virus. *J. Med. Virol.* 49, 83–86. [https://doi.org/10.1002/\(SICI\)1096-9071\(199606\)49:2](https://doi.org/10.1002/(SICI)1096-9071(199606)49:2).
- Soldateschi, D., Dal Maso, G.M., Valassina, M., Santini, L., Bianchi, S., Cusi, M.G., 1999. Laboratory diagnosis of toscana virus infection by enzyme immunoassay with recombinant viral nucleoprotein. *J. Clin. Microbiol.* 37 (3), 649. <https://doi.org/10.1128/jcm.37.3.649-652.1999>.
- Sui, L., Sun, P., Liu, N., Zhao, Y., Zhao, Y., Zheng, J., Liu, Q., 2025. The public health threat of emerging phenuiviruses. *One Health* 20, 101055. <https://doi.org/10.1016/j.onehlt.2025.101055>.
- Sword, T.T., Abbas, G.S.K., Bailey, C.B., 2024. Cell-free protein synthesis for nonribosomal peptide synthetic biology. *Front. Nat. Prod.* 3, 1353362. <https://doi.org/10.3389/FNTPR.2024.1353362/FULL>.
- Thirion, L., Pezzi, L., Pedrosa-Corral, I., Sanbonmatsu-Gamez, S., de, Lamballerie, X., Falchi, A., Perez-Ruiz, M., Charrel, R.N., 2021. Evaluation of a trio toscana virus real-time RT-PCR assay targeting three genomic regions within nucleoprotein gene. *Pathog. (Basel Switz.)* 10 (3), 1–11. <https://doi.org/10.3390/PATHOGENS10030254>.
- Woelbern, A.M., Ramm, F., 2024. Circumventing the impossible: cell-free synthesis of protein toxins for medical and diagnostic applications. *Int. J. Mol. Sci.* 2024 25 (24), 25. <https://doi.org/10.3390/ijms252413293>.