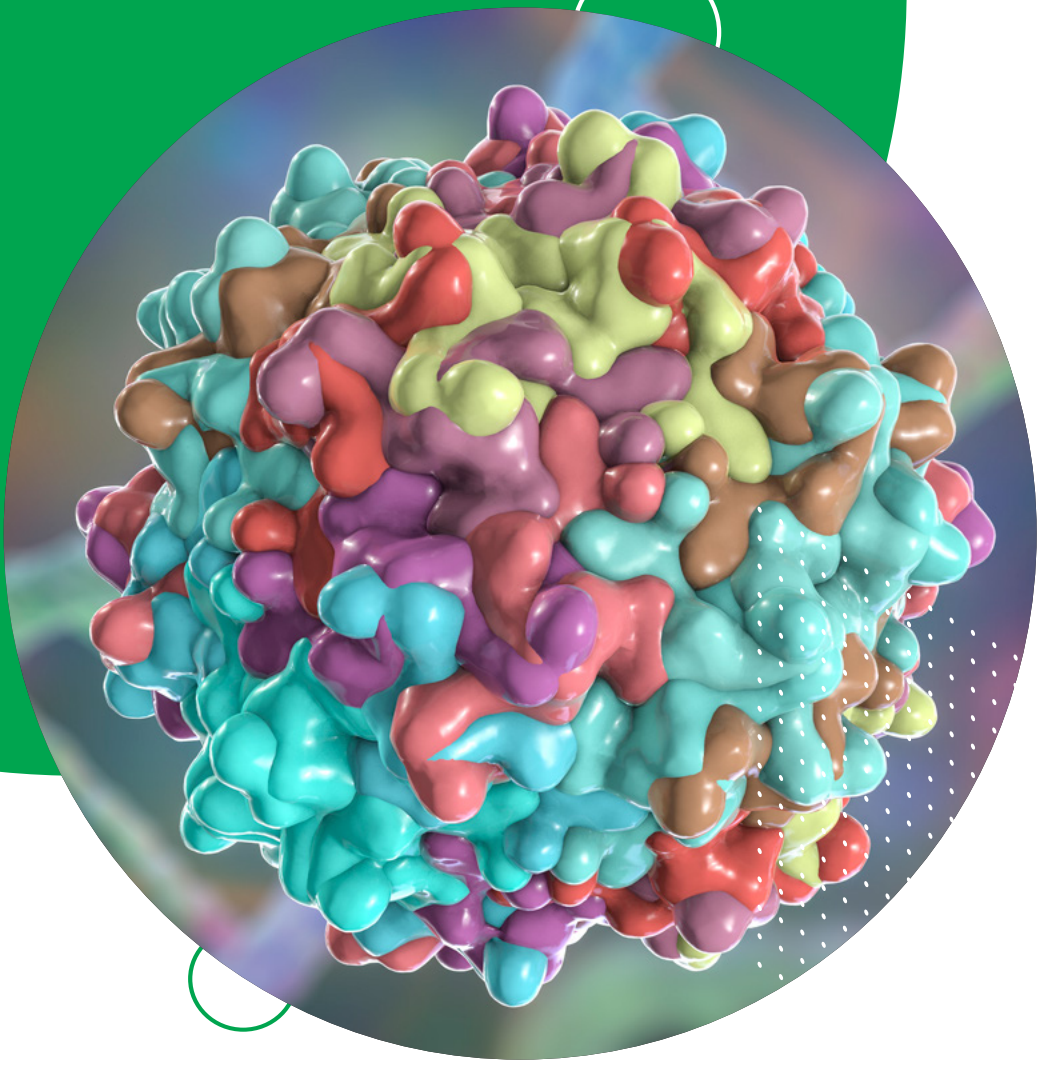
FROM CONCEPT TO CURE: USING AAV IN GENE THERAPY



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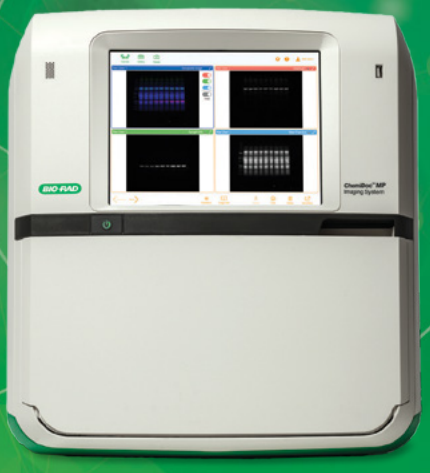

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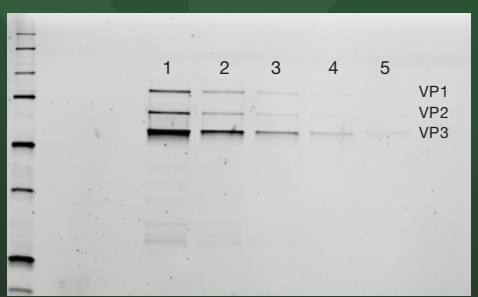
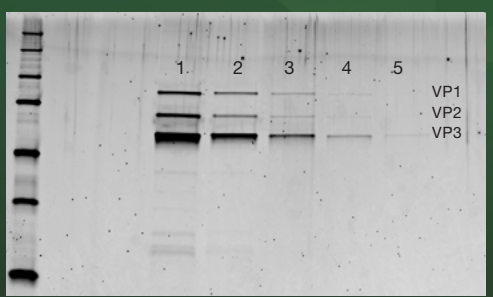


SYPRO Ruby Staining



Lane 1: 3.0 x 10^{10} VG
Lane 2: 1.0 x 10^{10} VG
Lane 3: 3.3 x 10^{9} VG
Lane 4: 1.1 x 10^{9} VG
Lane 5: 3.7 x 10^{8} VG

To test the sensitivity of different stains, a serial dilution of purified AAV was run on TGX Stain-Free Gels. The Stain-Free gel was activated with UV light prior to imaging. For SYPRO Ruby staining, the gel was fixed for 30 minutes, stained overnight, and then de-stained for 1 hour. Both gels were imaged on the ChemiDoc MP (Stain-Free: 90 second exposure, SYPRO Ruby: 2.7 second exposure).

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AAV VECTORS IN GENE THERAPY: CHALLENGES AND CONSIDERATIONS

Gene therapy treats or prevents disease by correcting or supplementing underlying genetic defects. After researchers identify a disease-causing mutant gene, they can restore its function by introducing a healthy gene copy or normalizing the levels of the dysfunctional gene product. Because viruses efficiently insert nucleic acids into host cells upon infection, they are attractive vehicles for gene delivery. Researchers previously tested many viruses as gene delivery agents for gene therapy. However, most of them were unsuitable for clinical applications due to their high immunogenicity and propensity to cause cancers.[1] To bring gene therapy to the clinic, scientists now use adeno-associated viruses (AAVs) for safe and effective gene delivery.

AAV Assembly

AAVs are small, single-stranded DNA viruses commonly used as gene delivery vehicles. Researchers work with AAV strains that have had most of their viral genes removed to make room for nucleic acid cargo. To develop an engineered recombinant AAV (rAAV) vector carrying a transgene for gene therapy, researchers use different plasmids and cell lines for assembly and production. The first plasmid contains the human gene of interest flanked by inverted terminal repeats (ITRs), DNA sequences that facilitate transgene packaging into the vector. The second plasmid carries *Rep* and *Cap* sequences that produce AAV capsid proteins. The wild-type (WT) AAV requires helper viruses, such as adenovirus or herpes simplex virus, for replication and infection. As a substitute for these additional viruses, the third helper plasmid provides adenovirus *E4*, *E2a*, and *VA* genes for reproduction. Co-transfecting these plasmids into host cell lines such as human embryonic kidney 293 (HEK293) or HeLa cells generates rAAV particles. Alternatively, many gene therapy workflows incorporate producer cell lines that express *Rep*, *Cap,* and viral helper genes instead of using plasmids. A typical fully-formed rAAV gene delivery particle carries a mammalian promoter and the ITRs flanking the transgene of interest encased in a viral capsid. In addition to the human and viral genes, researchers must consider several other factors such as target cell type, the promoter, and safety and efficacy of the AAV vector when designing rAAVs for gene therapy.[2]

AAV Serotype Selection and Optimization

There are eleven serologically-detectable AAV subtypes that infect different cell types based on the capsid proteins and cell-surface receptors present. Determining which cell types or tissue will receive the rAAV product is a crucial design criterion in gene therapy applications. To enter host cells, an AAV interacts with cell surface receptors. Changes in the AAV capsid sequence alter its sugar-binding preference, directly affecting its infectivity. While the AAV2 serotype is preferable for gene therapy because it infects most human cells, researchers have optimized other serotypes for organ-specific applications. For example, AAV1 and AAV9 infect skeletal and cardiac muscles, whereas AAV8 is suitable for DNA delivery in the liver. However, certain cell types remain inaccessible for gene therapy with wild-type AAV serotypes, so researchers are engineering new serotypes to enhance cell targeting.[3]

Promoter characterization is another crucial step in AAV vector design. Depending on the target cell type, researchers often choose strong, constitutively-expressing promoters that express genes at high levels. Utilizing promoters that are active in most cell types is a common gene therapy strategy; however, certain cell types silence these promoters. For example, the cytomegalovirus (CMV) promoter expresses in most mammalian cells, but does not work in the brain.[4] Researchers have circumvented this problem by using target-restricted, tissue-specific promoters.[5]

Enhancing AAV Efficacy and Safety

AAV vectors are preferred in gene therapy research due to their lack of immunogenicity compared to other viruses. Despite this, AAVs can trigger immune responses in patients for several reasons. First, people may have prior exposure, so AAVs can trigger a memory response involving neutralizing antibodies and T cells. Determining a therapeutic dose in patients that carry immune signatures against this virus is a formidable challenge in AAV gene therapy. The immune system can also detect the engineered transgene sequence as a foreign agent, activating immune pathways. Clinicians tackle the immunogenicity problem by either screening pre-existing neutralizing antibodies against AAV serotypes in patients or temporarily clearing antibodies from the patient's blood circulation.[6]

Please see references on page 7

ADDRESSING DEVELOPMENTAL HURDLES IN AAV GENE THERAPY

With significant advancements in molecular biology technologies and promising results in clinical trials, recombinant adeno-associated vectors (rAAVs) are gaining popularity for treating human genetic diseases. However, validating rAAV assembly and production at each workflow step creates barriers for bringing the technology to the clinic in a cost-effective manner. Researchers aim to streamline quality control steps that evaluate rAAV yield, purity, and potency to make these therapeutics safer and cost-effective.[1]

Capsid formation and genome packaging occur during rAAV production. Removing genetic contaminants and empty or partial capsids is critical to enhance infectivity and protein production as well as minimize immunogenicity and off-target effects. Researchers exploit numerous analytical techniques to obtain high yields of properly assembled rAAV particles, which enhances gene therapy potency.[1,2]

Characterizing the rAAV Genome

rAAVs often harbor unwanted DNA fragments that become encapsulated during assembly in production cell lines. Using next-generation sequencing, researchers detect unwanted genomic fragments and verify the accuracy of the genomic material in rAAV. Once researchers obtain pure rAAV particles, they determine the viral concentration as vector genomes per mL, which establishes the correct therapeutic dose. Quantitative PCR (qPCR) is a common method to quantify viral genome concentration. However, even with good primer design, the secondary structure in the ITR regions results in PCR efficiencies outside acceptable ranges.[3] Therefore, establishing a standard curve proves an impossible challenge for researchers. Droplet Digital™ PCR (ddPCR™) is a robust alternative to qPCR that allows researchers to quantify absolute levels of AAV genomes with higher sensitivity and no reliance on a standard curve. Using this technique, template amplification occurs after a sample is partitioned into thousands of sub-nanoliter sized droplets and amplified to endpoint.[4] Using ddPCR, researchers also monitor multiple genes simultaneously to determine whether the genome is intact.

Determining Proper rAAV Assembly

Transfection of the aforementioned DNA plasmids can produce empty or partially filled viral vectors that do not carry the transgene of interest. Various quality control methods such as gel and capillary electrophoresis, electron microscopy, and spectrophotometry facilitate rAAV characterization.

Quantifying the number of fully-assembled rAAV particles and capsid protein subtypes are critical safety and efficacy quality control measures in both preclinical and clinical stages.[1,5] Quality control steps measuring rAAV assembly, purity, and vector protein identification require gel electrophoresis. For example, a typical AAV capsid contains proteins such as viral protein (VP) 1-3, and the ratio of these proteins in the capsid directly influences the vector potency by increasing capsid stability and infectivity. Separation of these proteins by SDS-PAGE electrophoresis is a common method for determining VP subtype ratios. Moreover, gel electrophoresis detects empty capsids without genomic material.[5] To visualize proteins of interest, researchers incorporate the Bio-Rad™ ChemiDoc MP Imaging System into electrophoresis workflows. Using this instrument, they can process multiple gels and blots and visualize protein gels without using stains, which improves the speed and throughput of large-scale productions. With the accompanying Image Lab software with optional security edition to enable 21 CFR part 11 compliance, researchers easily quantify the levels of each protein subtype in the viral capsids.

While gel electrophoresis is a robust tool, it cannot detect some capsid proteins that are expressed at low levels. Therefore, researchers also incorporate a capillary electrophoresis method, allowing them to separate and quantify a range of peptides based on their charge. A spectrophotometric detector provides an accurate readout of the proteins.[4,6]

In addition to identifying capsid protein subtypes, morphological characterization of the fully-assembled rAAV particle is an essential quality control step. Because researchers produce AAV particles in living systems, post-translational modifications of the VP can alter viral vector potency and immunogenicity. By incorporating electron microscopy and mass spectrometry methods in quality control workflows, researchers can evaluate AAV capsid protein variability at a structural level in different batches.[4]

The End Game - Patient Monitoring

Preclinical assessment of rAAV production in animal models receiving gene therapy allows researchers to test their constructs' expression in vivo. Researchers harvest patient blood or other biological fluids that carry rAAV RNA and quantify their levels using qPCR or ddPCR. In cases of systemic gene therapy administration, protein product quantification using gel electrophoresis is also possible in clinical trials.[7]

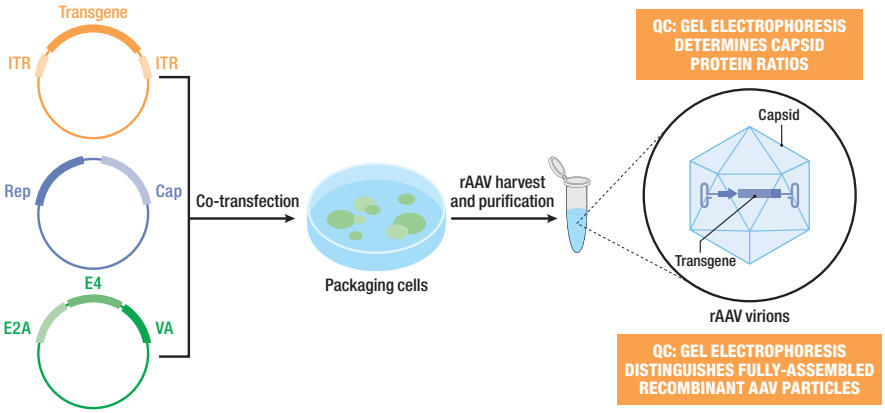
Please see references on page 7

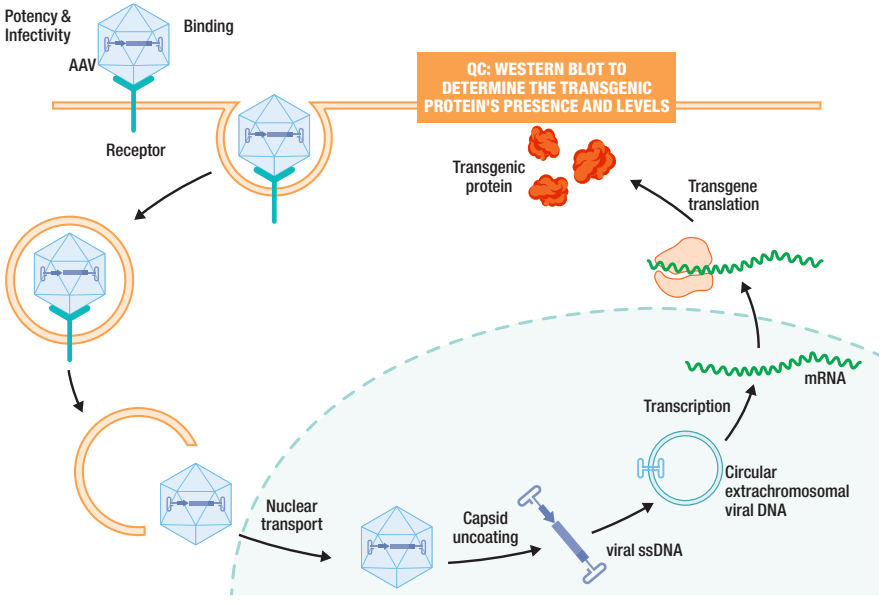
AAV CHARACTERIZATION: QUALITY TESTING WITH ELECTROPHORESIS

Gel electrophoresis is an important quality control assay in recombinant AAV (rAAV) assembly and production. The Bio-Rad ChemiDoc MP Imaging System facilitates the identity of AAV capsid surface protein types, assembled rAAV VP ratio, and final gene products in quality control (QC) steps.

AAV Vector Design and Assembly for Gene Therapy

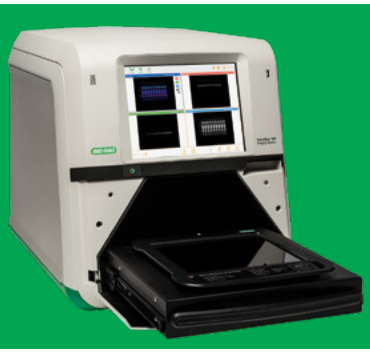
Co-transfecting the transgene of interest, *Rep/Cap,* and helper plasmids into producer cell lines is the first step in rAAV manufacturing. Together, these plasmids produce functional rAAV virions carrying a human transgene ready to infect a variety of mammalian host cells in preclinical and clinical models.





Recombinant AAV Infection and Protein Production

Once in the host body, an rAAV gene delivery vector infects its target cell. The vector first binds to its cognate cell surface receptors where internalization through the cell membrane facilitates cell entry. Subsequent trafficking steps bring the DNA cargo to the nucleus where the linear single-stranded DNA circularizes forming self-complementary extrachromosomal DNA. These transcriptionally-active structures produce proteins and restore cellular functions in gene therapy applications.



Quality Control with the ChemiDoc MP Imaging System

An all-in-one imaging system detects protein gels and western blots precisely and reproducibly in recombinant AAV gene therapies.

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- Easy viral capsid ratio determination with Image Lab analysis software

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CURING GENETIC DISEASES WITH AAV-BASED THERAPEUTICS

In the late 90s, Jesse Gelsinger, a volunteer in a gene therapy trial for a rare metabolic disorder, suffered a fatal immune response to the delivered adenovirus therapeutic.[1] Because of this outcome, researchers suffered a major setback in developing human gene therapies, which stagnated progress for a decade. However, the gene therapy field experienced a renaissance in the last decade due to unprecedented advances in AAV technology. Precise control of gene delivery, immune system modulation, and progress in human genomics allowed researchers to develop two FDA-approved gene therapies for human genetic disorders with many more in the pipeline.[2]

Fighting Blindness

Retinal dystrophies are heterogeneous ocular neurodegenerative diseases causing severe visual defects and blindness. Luxturna is the first AAV-based gene therapy approved in the US for biallelic 65 kDa retinal pigment epithelium (RPE)-specific protein (RPE65) mutation-associated retinal dystrophy.[3,4]

RPE65 is an essential gene for normal vision in the human retina. When light enters the eyes, it changes the molecular conformation of 11-cis-retinal, a form of vitamin A, to all-trans-retinal, generating electrical excitation in retinal neurons. Retinal neuron activation sends the visual information to downstream brain neurons. The RPE65 protein converts all-trans retinal back to 11-cis retinal to reset the visual cycle. Mutations in *RPE65* reduce or obliterate RPE65 activity, impairing vision. Luxturna provides a normal copy of *RPE65* via an AAV2 vector targeted to the patient's retina, which restores vision loss.[3,4]

Patients typically receive the treatment in each eye on separate days through subretinal injection. To limit potential immune reactions, physicians prescribe patients oral prednisone. Researchers measure the therapy's success by determining the patient's ability to navigate an obstacle course at various light levels throughout the year after receiving treatment. Luxturna receivers showed huge improvements in their ability to complete the course at low light levels compared to the control group.[3,4] While Luxturna revolutionized the gene therapy field, physicians can offer it only to patients who still have viable retinal cells.

The success of Luxturna and Zolgensma provides significant opportunities to develop gene therapy products for other genetic disorders.

Prolonging the Lives of SMA Patients

Spinal muscular atrophy (SMA) is a rare genetic disorder caused by a faulty survival motor neuron 1 (*SMN1*) gene. SMN1 protein is crucial for the survival and function of motor neurons that control muscles. Without functional SMN1 protein, motor neurons die rapidly, impeding basic motor functions, including breathing and swallowing. SMA Type 1 is the most severe disease form—symptoms appear within the first six months of life and most afflicted children die before the age of two.[5]

Zolgensma is a gene therapy specifically for SMA Type 1 where an intravenous infusion of an AAV9 vector carrying a healthy copy of SMN1 prolongs motor neuron survival. A single dose is sufficient to restore SMN1 protein levels and motor functions. Patients that received a high dose of Zolgensma in clinical trials did not require permanent ventilation, unlike patients not receiving the therapy. Most infants in the experimental cohort held their heads erect, sat without support for more than ten seconds, and could stand. Zolgensma successfully prolonged survival in infants. Life expectancy for SMA type I is less than 2 years, whereas children who received Zolgensma are now six years old. The therapy is currently available to patients in 38 countries.[6,7]

The success of Luxturna and Zolgensma provides significant opportunities to develop gene therapy products for other genetic disorders. More than 50 clinical trials are already underway to benefit patients suffering from rare genetic diseases such as Duchenne muscular dystrophy, X-linked myotubular myopathy, and several lysosomal storage disorders.[2]

Please see references on page 7

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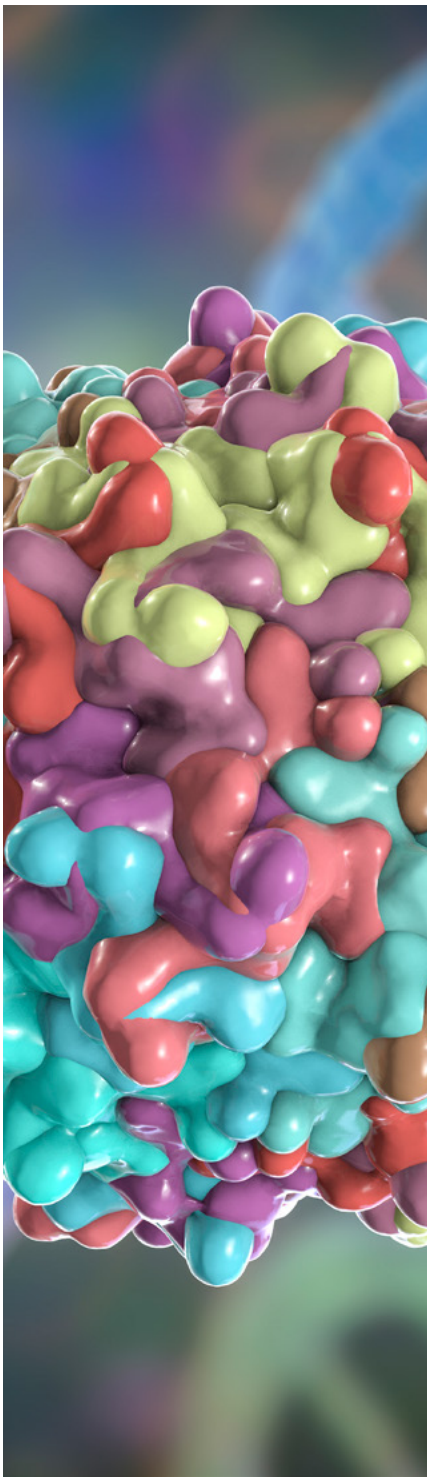

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