evropský sociální fond v ČR
EVROPSKÁ UNIE
MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY
OP Vzdělávání pro konkurenceschopnost
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

Název: Cloning, characterization and isolation of GFP protein

Školitel: Ana Maria Jimenez Jimenez

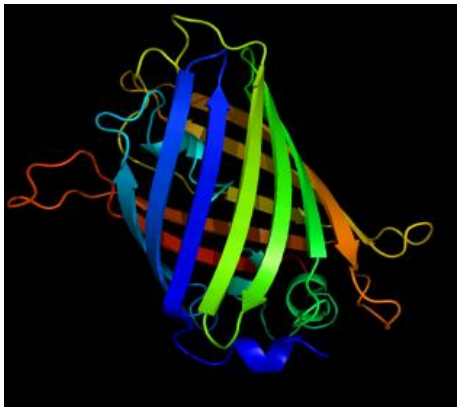
Datum: 04.10.2013

Reg.č.projektu: CZ.1.07/2.3.00/20.0148

Název projektu: Mezinárodní spolupráce v oblasti "in vivo" zobrazovacích technik

GFP - Green fluorescent protein

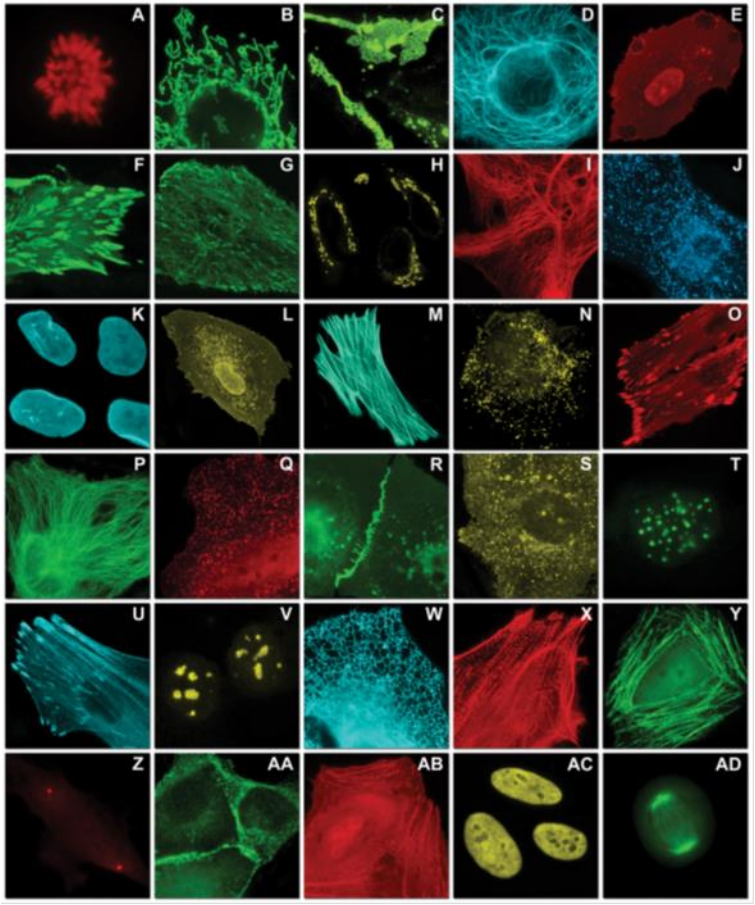
- GFP was isolated from the jellyfish *Aequorea victoria*.
- This protein is composed of 238 amino acid residues (26.9 kDa).
- Exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range.
- In cell and molecular biology, the GFP gene is frequently used as a reporter of expression.
- The GFP gene has been introduced and expressed in many bacteria, yeast, fungi, fish, plant and mammalian cells.



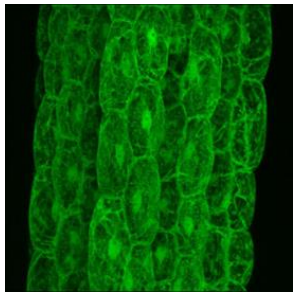
Green fluorescent protein

- Osamu Shimomura got the Nobel Prize in Chemistry (2008) for the discovery and development of the green fluorescent protein.
- GFP is commonly used molecular imaging tool in Biology, Chemistry, Genetics and Medicine.
- GFP can be attached to other proteins allowing scientist to monitor processes in living organisms.
- The enormous flexibility as non-invasive marker in living cells allows for numerous applications, such in cancer studies.

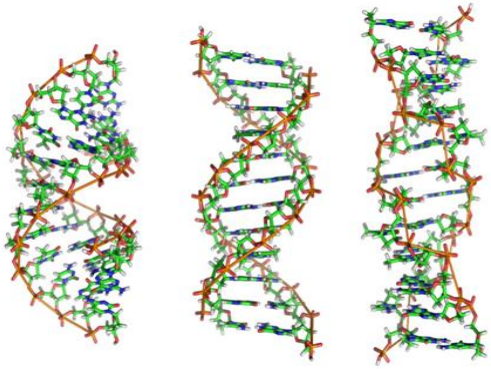
GFP Types

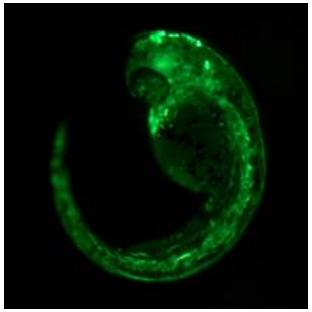


- Fluorescent proteins enable the creation of highly specific biosensors to monitor a wide range of intracellular phenomena.
- Mutagenesis of *A. victoria* GFP has resulted in fluorescent proteins that range in colour from blue to yellow.



MOLECULAR CLONING

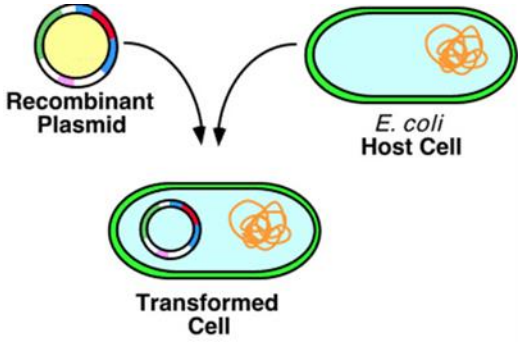




GFP in pGLO plasmid

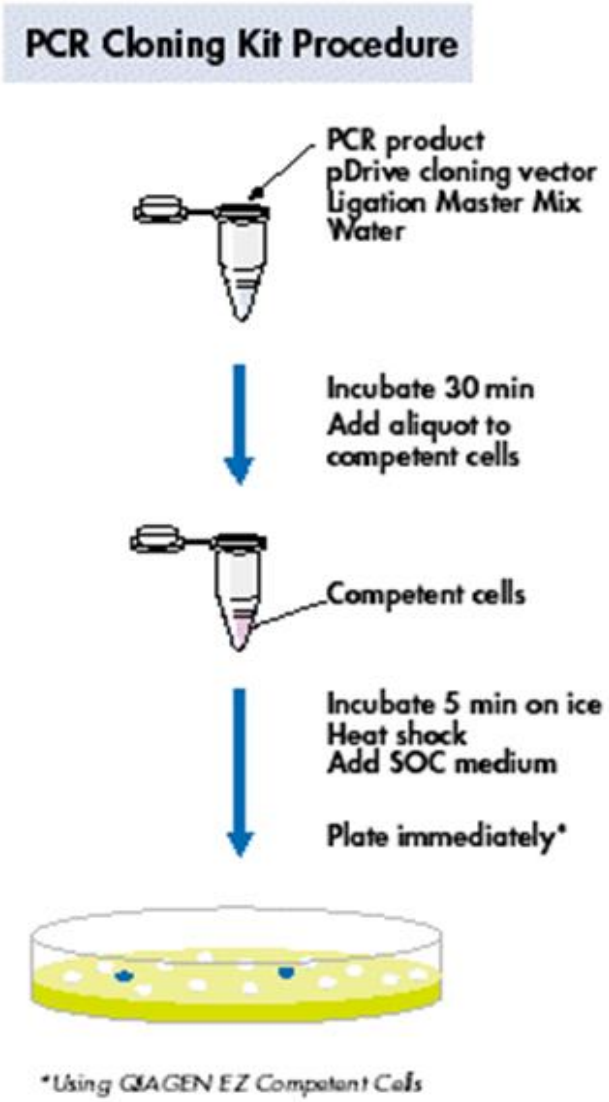
- The pGLO plasmid is used in Biotechnology.
- The plasmid contains the green fluorescent protein (GFP) and the ampicillin resistance gene.
- The GFP gene is expressed in the presence of arabinose, which makes the transgenic organism shows fluorescence under UV light.
- GFP can be induced in bacterias which contains the pGLO plasmid and grows on arabinose and ampicilin plates.

pGLO™ Bacterial Transformation Kit (Biorad)



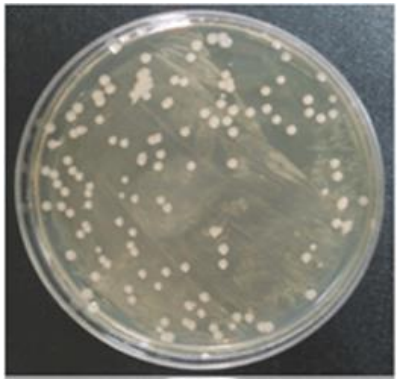
Cloning

- Add 2 µl of the pGLO plasmid into vial of chemical competent cells.
- Incubate on ice 30 minutes.
- Heat shock the cells for 45 seconds at 42°C.
- Transfer the tubes to ice for 3 minutes.
- Add 250 µl of SOC medium.
- Shake the tube horizontally (200 rpm) at 37°C for 90 minutes.
- Spread 50 µl and 200 µl from transformation on selective plate (ampicilin+arabinose)
- Incubate overnight at 37 °C.



Results of Cloning

pGLO Cloning

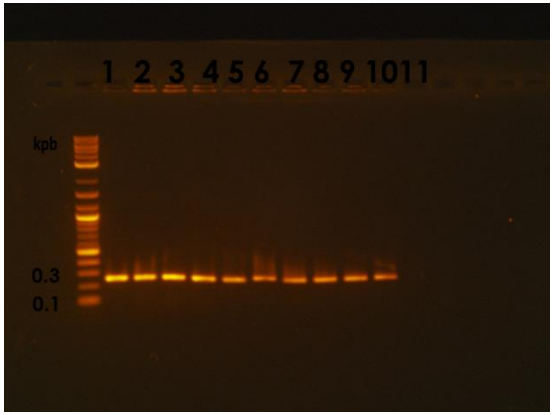


LB+ampicilin+arabinose

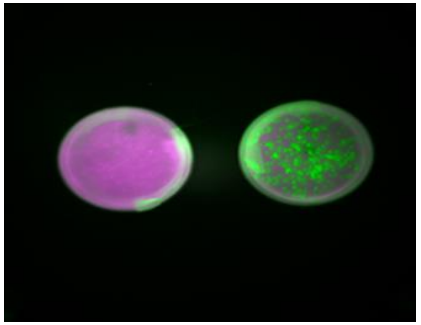
A

B

PCR Screening



In-vivo Xtreme



Analysis of Positive Clones

- Pick 10 colonies and make **PCR Screening**

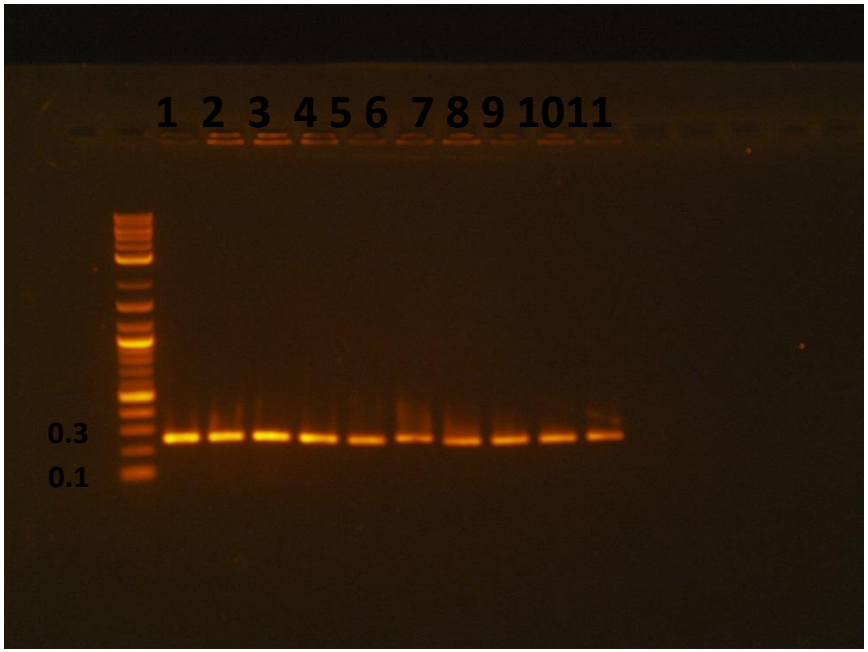
PCR (GFP Plasmid) 35x

4 min 95 °C
1 min 95 °C
30s 50 °C
30s 72 °C
10 min 72°C
10 min 72°C
10 min 10 °C

PCR product **272bp**

Electrophoresis
1% AgarGel
Ethidium Bromide 5 µl/100µl
TAE Buffer, 100v
60 min, UV

PCR
1-10. Colonies
11. Negative control



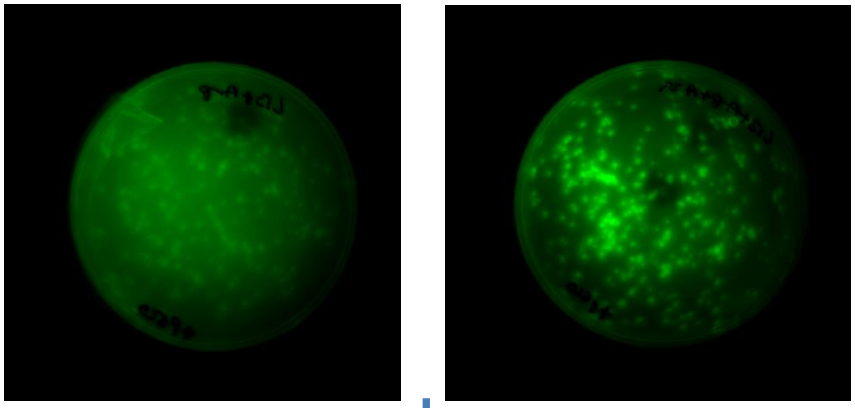
All colonies were positive transformants!!

pGLO GFP - fluorescence detected

In-vivo Xtreme

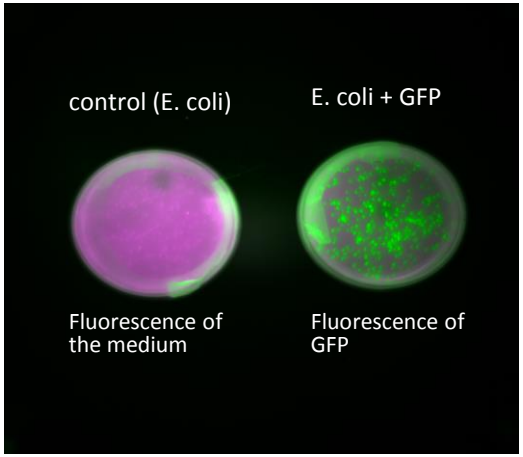
Control (E. coli)

E. coli + GFP



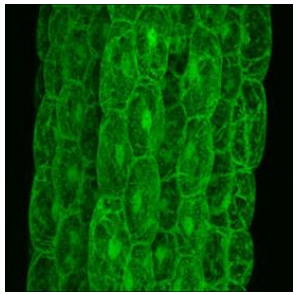
In vivo Imaging studies

Unmixing software

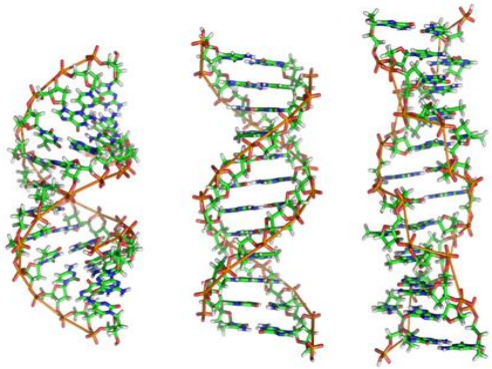


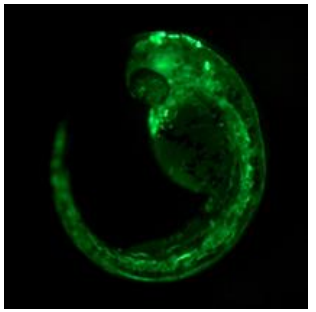
Autofluorescence of LB medium

Green flourescence of GFP



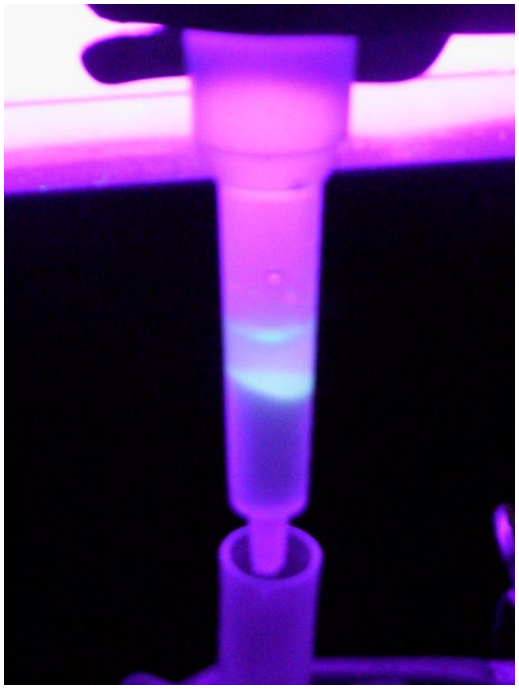
ISOLATION OF GFP PROTEIN





Bacterial Lysis and Protein Chromatography

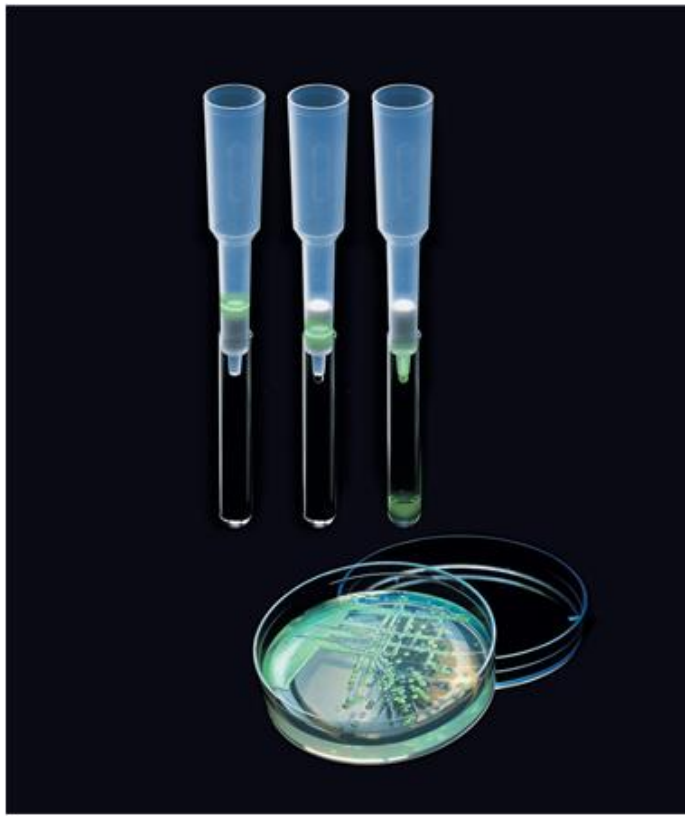
- The positive transformants were grown in LB broth with 100 mg/liter ampicillin and 0.2% arabinose.
- Shaking the culture overnight at 32 °C.
- Enzymatic lysis of the bacterial cell wall with10 mg/ml of lysozyme and freezing at -80°C.
- GFP was purified from the bacterial lysate using hydrophobic interaction chromatography (HIC) columns (Macro-Prep® Methyl HIC Column, Biorad).
- The protein elution was made with TE buffer.



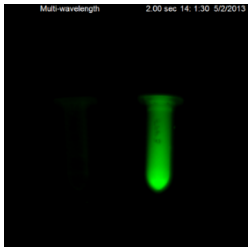
Chromatography Column using the UV light

Protein isolation

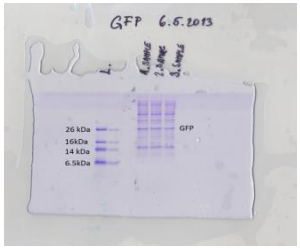
Protein Chromatography



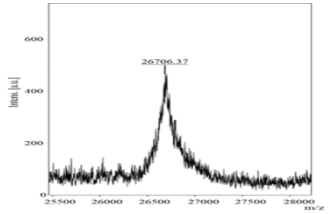
In-vivo Xtreme



SDS-PAGE Electrophoresis



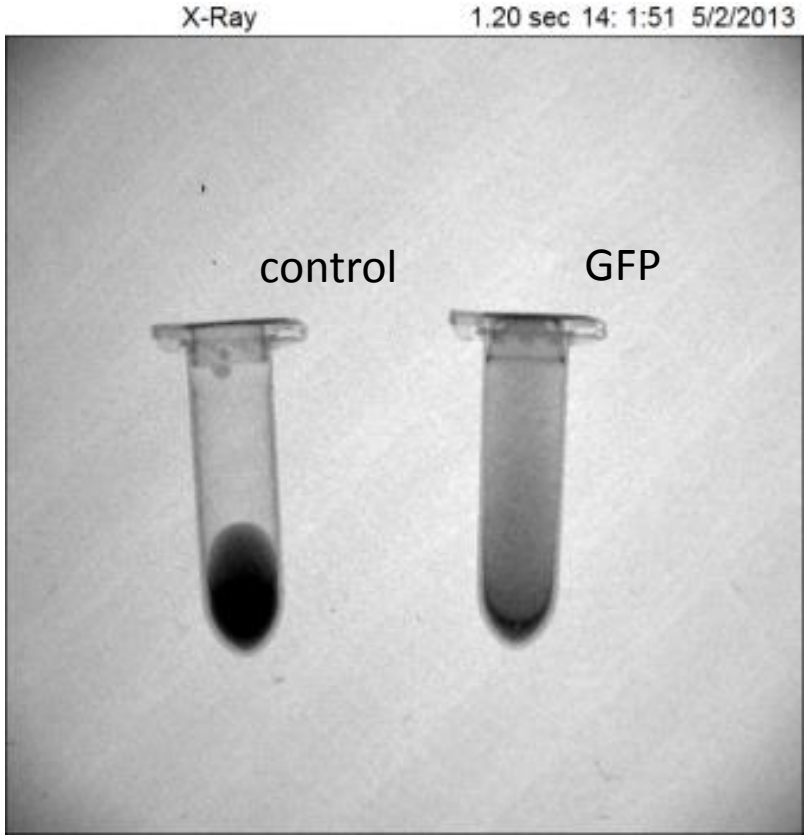
MALDI-TOF

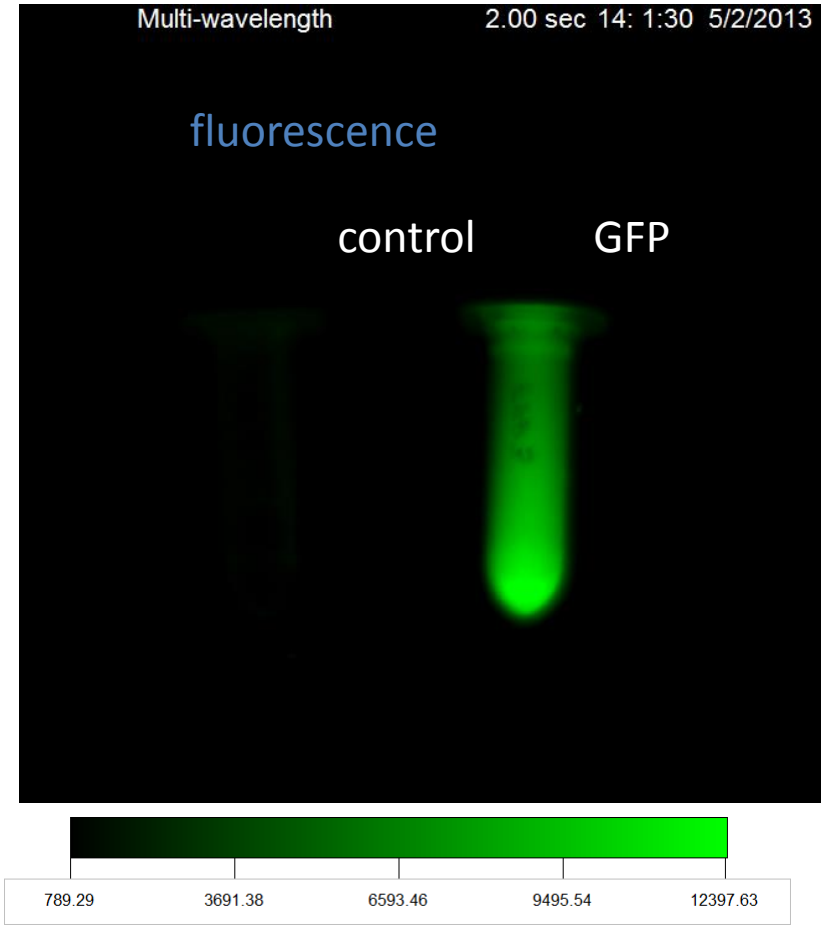


GFP protein

Fluorescence Detected

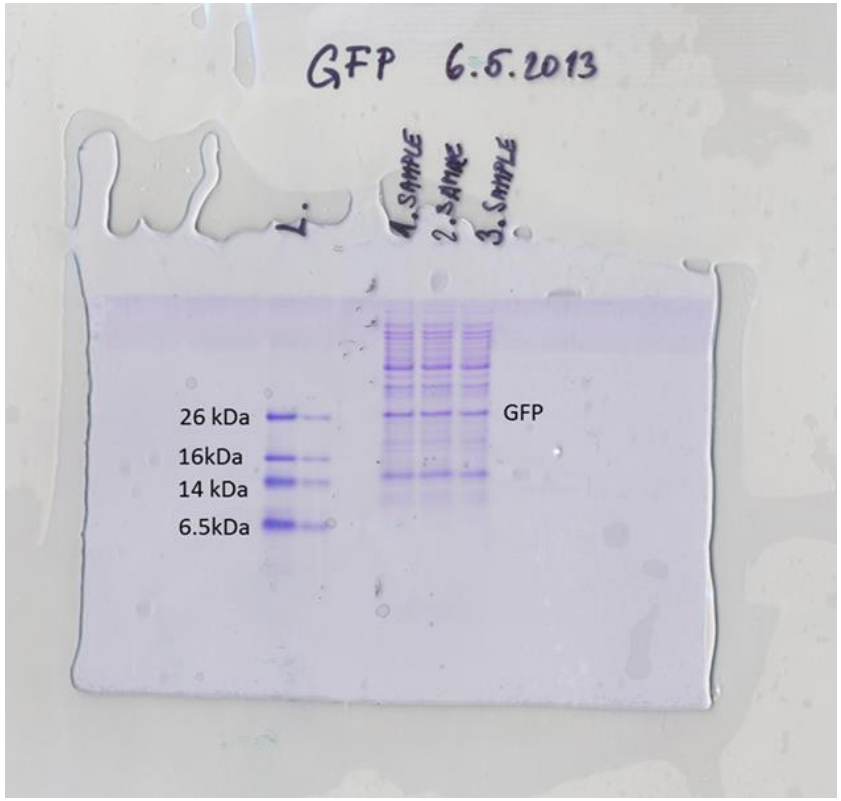
In-vivo Xtreme





Sds-page electrophoresis

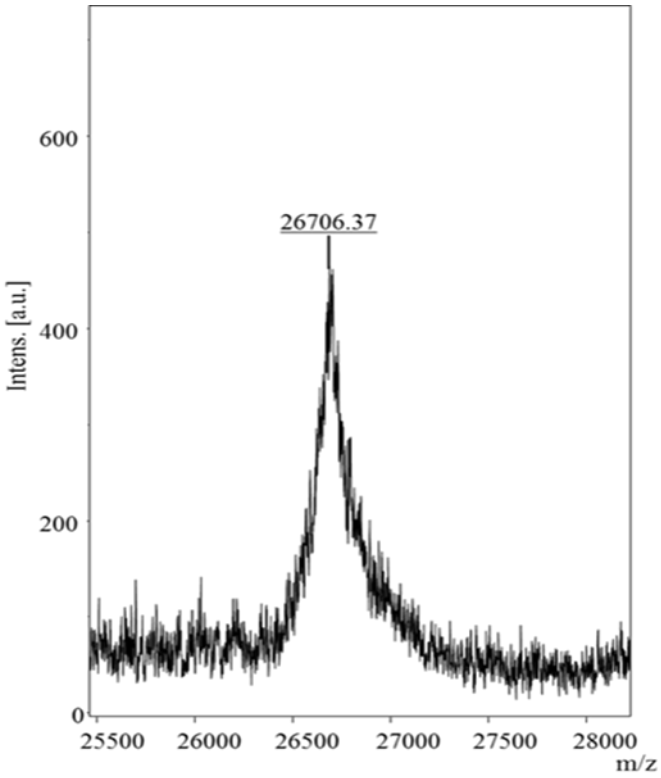
- SDS-PAGE, is a technique to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility. Mobility is a function of the length, conformation and charge of the molecule.
- The result in acrylamide gel were correct, the expected atomic mass of the full GFP was 26kDa.



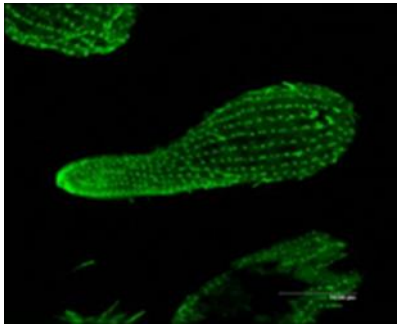
PGE gel

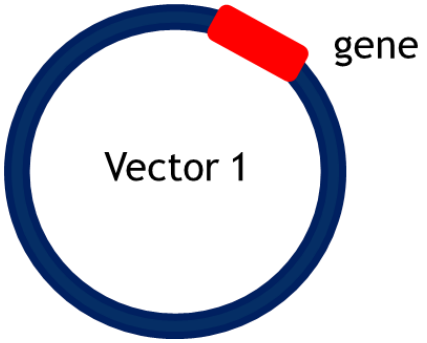
GFP Espectra MALDI-TOF/TOF

- The matrix used in the MALDI method was α-cyano-4-hydroxycinnamic acid (CCA).
- The matrix was prepared in TA30. The MS spectra were acquired by averaging 20 sub spectra from a total of 500 shots of the laser.
- The results in MALDI were correct, the expected size of the full GFP was 26700 Da.

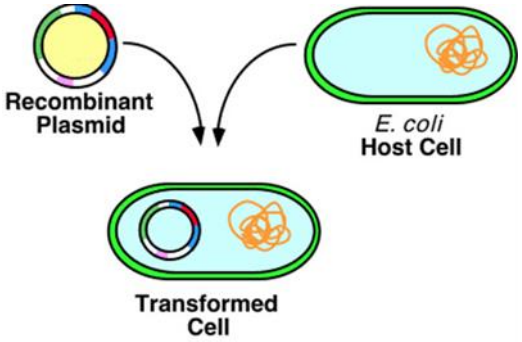


Spectra of Green Fluorescent Protein by MALDI-TOF

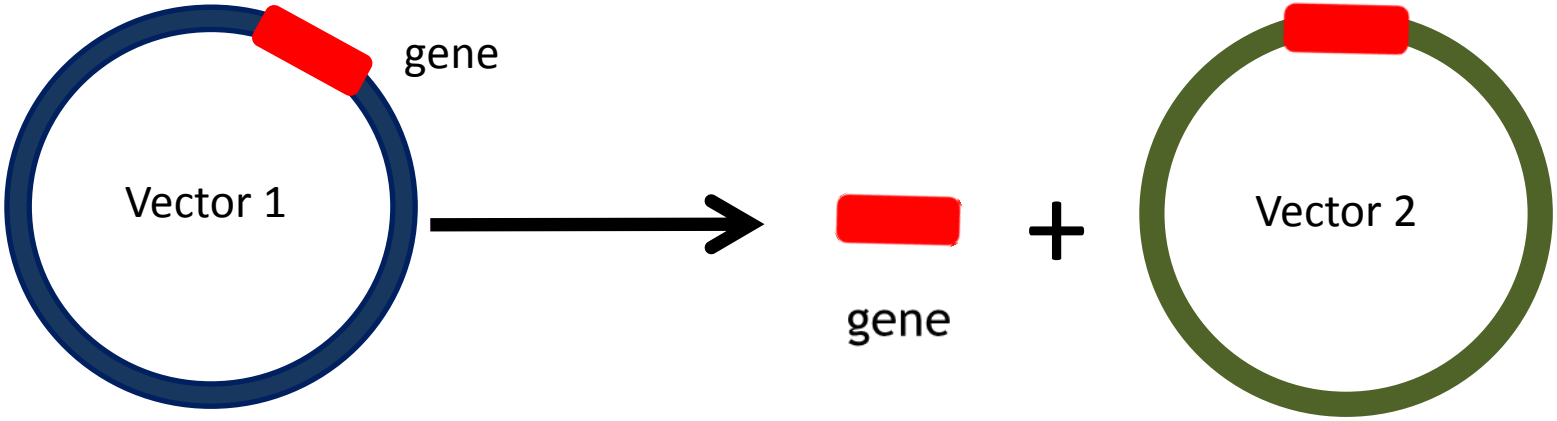



gene
Vector 1

SUBCLONING

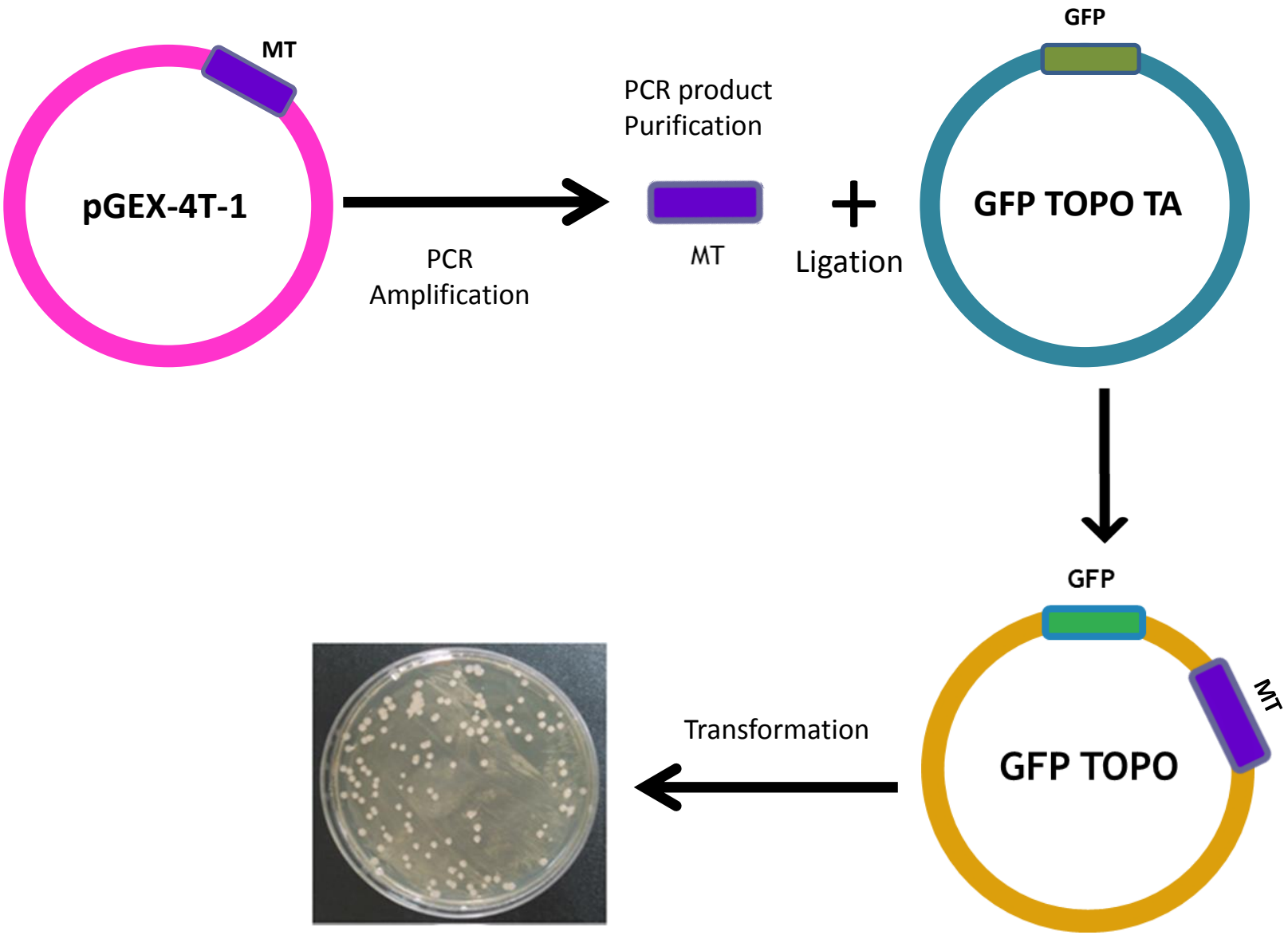

Recombinant Plasmid
E. coli Host Cell
Transformed Cell

Subcloning OF METALLOTHIONEIN GENE (MT) into GFP TOPO Vector



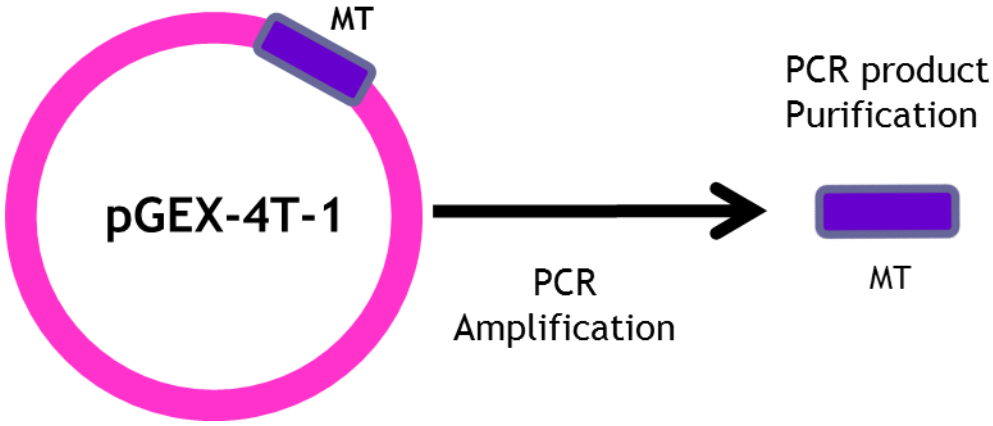
- Subcloning is a technique used to move a particular gene of interest from a parent vector to a destination vector in order to further study its functionality.

Subcloning protocol



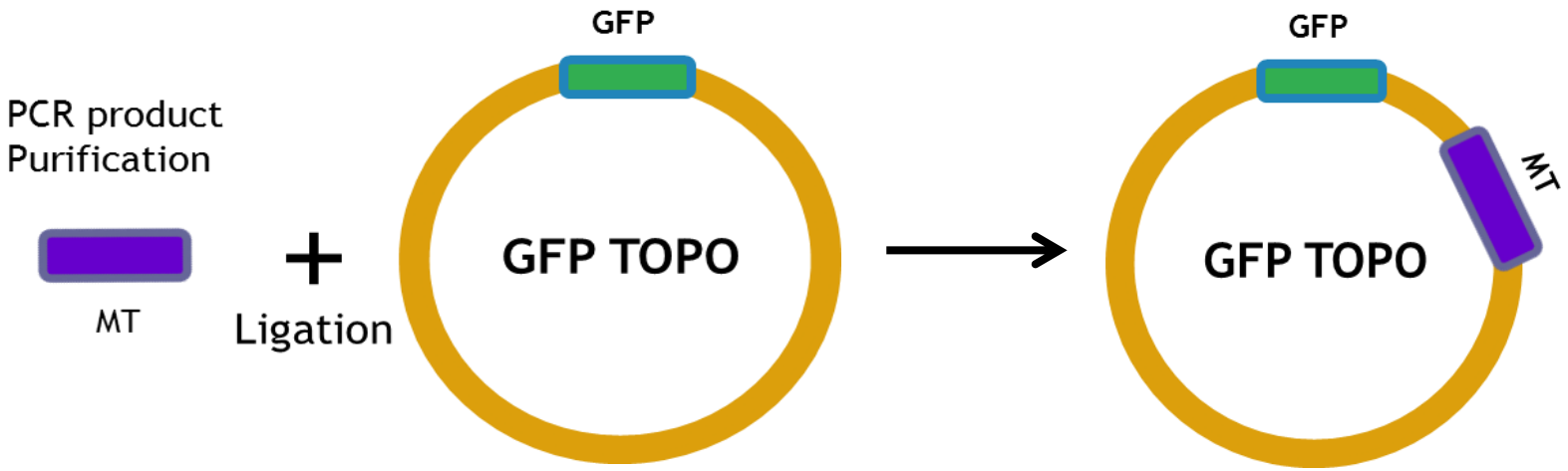
PCR AND PURIFICATION PCR PRODUCT

- Used like DNA template miniprep pGEX-4T-1-MT (100ng)
- Prepare a PCR with the primers for amplification of metallothionein gene
- Visualize by agarose gel electrophoresis
- Purification of PCR product. Purify 3 PCR product in the same MinElute column (Mini Elute PCR purification Kit, Quiagen)



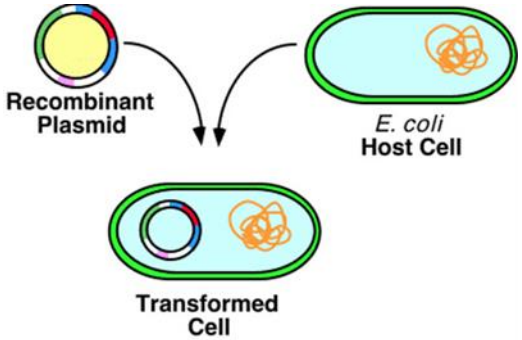
LIGATION

- Ligation of PCR purification product (Metallothionein) with GFP TOPO TA Vector.
- Relation Insert:Vector 5:1
- Reaction:
  Purification PCR product (3ng)
  Salt Solution 1µl
  GFP TOPO Vector (10ng) 1µl
  Deionized water to a final volume of 6µl
- Mix reaction gently and incubate the reactions 30 minutes at room temperature.



Chemical Transformation

- Add 2 µl of the GFP TOPO vector into a vial of chemically competent cells
- Incubate on ice for 30 minutes.
- Heat-shock the cells for 45 seconds at 42°C.
- Transfer the tubes to ice for 3 minutes.
- Add 250 µl of SOC medium.
- Shake the tube horizontally (200 rpm) at 37°C for 90 minutes.
- Spread 50-200 µl from transformation on a selective plate (LB agar+Amp).
- Incubate overnight at 37°C.
- Pick colonies for analysis (screening).

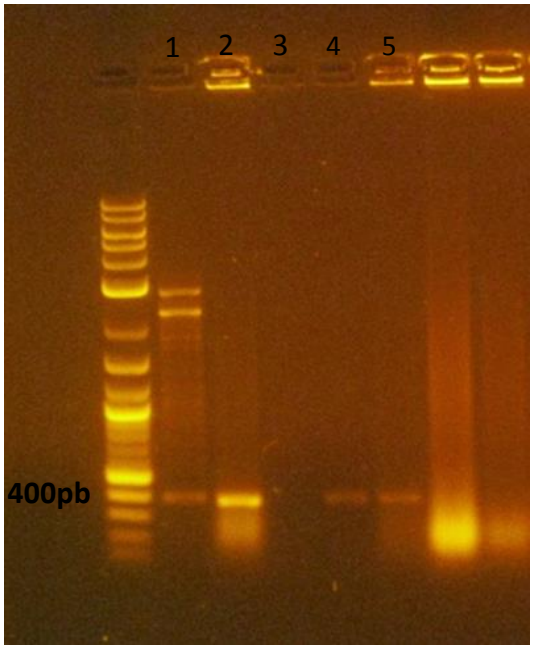


Analysis of Positive Clones

PCR (GFP Plasmid) 35x
4 min 95°C
1 MIN 95°C
30s 60°C
30s 72°C
10min 72°C
10min 72°C
10 min 10°C
PCR product 370pb

Primers: GFP forward
MT reverse

→ correct orientation

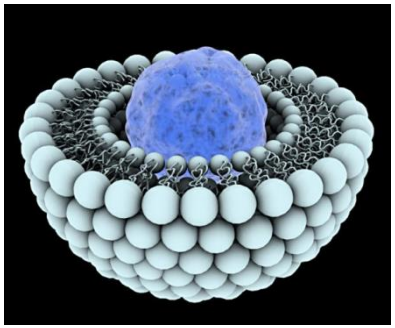


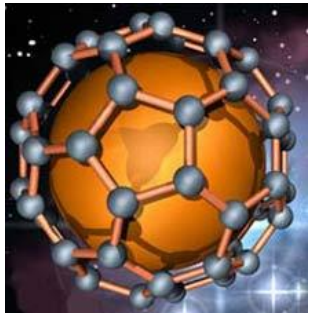
Electrophoresis
1% AgarGel
Ethidium Bromide 5 µl/100µl
TAE Buffer, 100v
60min, UV

1, 2, 4, 5 positive colonies ☺
MT gene into the GFP TOPO vector in the correct orientation

FUTURE WORK with gfp

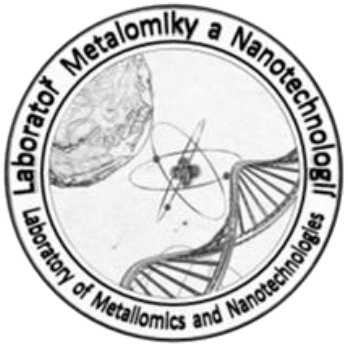
- Expression of metallothionein protein together with GFP protein (Ana)
- Isolation of big volume of GFP protein by Chromatography (Ana, Marketa, Roman)
- Liposome+GFP in vivo Imaging (Pavel, Iva)
- Nanoparticules+GFP (Marie)
- S.aureus+GFP (Ana)





Acknowledgements

- **Prof. Ing. René Kizek, Ph.D.**
- **Doc. Mgr. Vojtěch Adam, Ph.D.**
- **Mgr. Marie Konečná, Ph.D.**
- **Doc. RNDr. Pavel Kopel Ph.D.**
- **Ing. Iva Blažková**
- **Mgr. Dagmar Chudobová**
- **Lucie Dostálová**
- **Merlos Rodrigo Miguel Angel, DEA**
- **Ing. Kristýna Šmerková**
- **Mgr. Markéta Komínková**
- **Bc.Roman Guráň**



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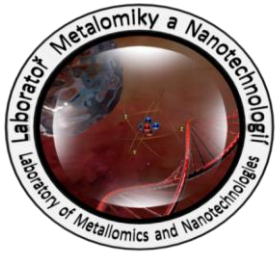
Mendel University in Brno

Thank you for your attention!



Reg.č.projektu: CZ.1.07/2.3.00/20.0148

Název projektu: Mezinárodní spolupráce v oblasti "in vivo" zobrazovacích technik

NLAB SYS