flavoflow

Lab Protocols & Safety

WET LAB

Contents

Media for *Flavobacterium columnare*, *Flavobacterium branchiophilum* and *Flavobacterium psychrophilum*

Component	Enriched cytophaga (V = 500 mL)	Medium 36 (V = 500 mL)
Tryptone	1 g	2.5 g
Meat extract	0.5 g	0.1 g
Yeast extract	0.5 g	0.25 g
Sodium acetate	0.1 g	0.1 g
Agar (optional)	7.5 g	5 g
Water	500 mL	500 mL

Media was sterilized in an autoclave.

Tryptone Yeast Extract Salts:

- 0.4% (w/v) tryptone
- 0.04% yeast extract
- 0.05% (w/v) $MgSO_4*7H_2O$
- 0.05% (w/v) $CaCl_2*2H_2O$
- 0.05% (w/v) D-glucose, pH 7.2

Chemically competent cell preparation

Items required: 5 mL LB-medium, 1.5 mL tubes, pre-cooled centrifuge, 100 mM of $CaCl_2$ solution

1. Inoculate 5 mL overnight-culture in LB-medium without antibiotics (for DH10B or BL21(DE3)) or with other strain-appropriate antibiotics.
2. Incubate samples to OD_{600} = 0.5 - 0.6 in a shaking water bath at 37 °C, 200 RPM.
3. Transfer 1 mL of culture into each of the 1.5 mL test tubes and spin down in a 4 °C centrifuge for 1 min at 9500 RCF. Discard the supernatant.
4. Carefully suspend each pellet in 500 µL of ice-cold 100 mM $CaCl_2$ solution and incubate on ice for 15-30 mins.
5. Centrifuge samples in a 4 °C centrifuge for 1 min at 9500 RCF and discard the supernatant.
6. Carefully suspend each pellet in 100 µL of ice-cold 100 mM $CaCl_2$. These precipitates are then used for transformation right away.

Chemical transformation

1. Take bacteria out of the 80 °C freezer or prepare chemically competent bacteria before transformation.

2. Turn on the UV sterilization function in the working box for about 15 minutes to sterilize the working environment before opening the test tube with the bacteria to avoid any risk of contamination.
3. Take out petri dishes with required medium and antibiotic out of the refrigerator.
4. Put 2 µL of plasmid into 50 µL of competent cells culture (or 20 µL ligation mixture into 100 µL of competent cells culture). Also, prepare as many of the transformation controls as possible:
   (a) Transform the bacteria without DNA - contamination control.
   (b) Transform the bacteria with a linearized digested vector - to check if DNA is properly digested.
   (c) Transform the bacteria with purified non-digested DNA - positive control, to check transformation efficiency.
5. Hold the samples on ice for 10-30 minutes.
6. Perform a heat shock for 1.5 min at 42 °C.
7. Incubate samples on ice for 5-10 minutes.
8. Suspend the bacteria with 1000 µL of LB (Luria Broth).
9. Incubate samples in the 37 °C heat block for 20-40 minutes.
10. Centrifuge cells for 3 minutes at 4400 RPM.
11. Discard the supernatant in one quick movement and resuspend bacteria in the remaining LB medium. Plate out this mixture on an LB petri plate with the correct antibiotic.
12. Incubate the plates overnight at an optimal temperature for selected bacteria.

Protein induction

1. Cultivate transformants overnight on a plate with LB broth.
2. Transfer a single transformant colony to a 12 mL flask with LB medium with an appropriate antibiotic concentration.
3. Incubate flask in a water bath at 37 °C until OD_{600} = 0.5.
4. Once OD_{600} = 0.5 is reached, transfer the flask to 4 °C for 10 min.
5. After incubation transfer 1 mL of bacterial culture into a clean 1.5 mL tube.
   (a) All collected tubes needs to be prepared in this way:
      i. Centrifuge sample at 13 000 RCF at room temperature for 1-2 min and discard the supernatant.
      ii. Add 50 µL of SDS loading dye (10%) and β-mercaptoethanol (10%) solution, resuspend the pellet.
      iii. Incubate for 10 min at 100 °C in a dry bath.
      iv. Store samples at -20 °C.
6. For induction, add 1 mM IPTG inducer into bacterial culture. Incubate flasks in a shak-

ing water bath under selected conditions and induction duration.
7. After induction, centrifuge the remaining bacterial culture volume at 4 °C, 4000 RPM for 10-15 min, carefully decant the supernatant making sure not to lose any of the precipitate or evidently more viscous fluid.
8. Pipette away any of the remaining supernatant.
9. Remaining precipitate is then stored at -20 °C or used further in a sonication process.

Cell lysis by sonication

1. Resuspend the bacterial cell biomass with 1.5 mL of resuspension buffer (Tris-HCl pH 8.0, 50 mM).
2. Sonication was carried out using the Bandelin Ultrasonic Disintegrator HD2070.
3. Sonication process parameters:
   (a) 30% power
   (b) 3 cycles of 30 s ON / 30 s OFF sonication.
4. After sonication, centrifuge the sample at 13 000 RCF at 4 °C for 1-2 min and resuspend with 1.5 mL of resuspension buffer.
5. Repeat the sonication process.
6. Once again centrifuge the sample at 13 000 RCF at 4 °C for 1-2 min.
7. Remove the supernatant, prepare a supernatant electrophoresis sample: 20 µL of supernatant + 20 µL of SDS loading dye (10%)/β-mercaptoethanol solution (10%).
8. Resuspend the precipitate with 200 µL of resuspension buffer.

Frozen Competent *E. coli* preparation

1. Inoculate bacterial culture in 12.5 mL of LB for 3 hours until OD_{600} = 0.5.
2. Inoculate 250 mL LB in a 1 liter flask with 12.5 mL of saturated culture and measure the OD_{600} in 1 hour and every half hour thereafter until it reaches the 0.6 to 0.7 range. Harvest the cells by spinning the culture in a centrifuge in five 50 mL tubes at 3000 RPM for 10 min at 4 °C.
3. Discard the LB and gently resuspend each bacterial pellet in 1 mL of ice cold 100 mM $CaCl_2$, collect pellets into one tube and add $CaCl_2$ to the final 12.5 mL volume. Spin again at 3000 RPM for 10 minutes at 4 °C.
4. Discard the supernatant and gently resuspend bacterial pellet in 12.5 mL of ice cold 100 mM $CaCl_2$. Hold overnight on ice.
5. Spin at 3000 RPM for 5-10 minutes at 4 °C and gently resuspend pellet in 5 mL of 4:1 $CaCl_2$:Glycerol.

Agarose Gel preparation (x%)

1. For the preparation of agarose gels TopVision Agarose Tablets were used (manufactured by Thermo Fisher Scientific).
2. Add the necessary amount of agarose tablets to the electrophoresis buffer based on the table below in order to prepare the desired agarose concentration gel.
    - Note: Use a flask whose volume is 2-4 times greater than the one of the solution being prepared.

Gel, %	1 tablet	2 tablets	3 tablets
1%	50 mL	100 mL	150 mL
1.2%	42 mL	83 mL	125 mL
1.3%	38 mL	77 mL	115 mL
1.5%	33 mL	67 mL	100 mL
1.8%	28 mL	56 mL	83 mL
2%	25 mL	50 mL	75 mL
2.5%	20 mL	40 mL	60 mL
4%	12.5 mL	25 mL	37.5 mL

3. Before heating, let the tablets sit in a buffer for around 4 minutes until tablets break down into a slurry. Mix the slurry to break up any remaining particles. Note:
    - Make sure the tablets break up completely. Heating will render non-dispersed agarose particles insoluble.
    - Note: Heating times should be adjusted according to the solution volume and the number of tablets.
4. Remove the flask from the microwave, mix gently to dissolve any remaining agarose particles.
5. Continue heating until the solution is clear and all particles are dissolved.
6. Remove the flask from the microwave oven, mix gently.
7. Let the solution cool down to 50-60 °C.
8. Add ethidium bromide (EtBr) to a final concentration of 0.2-0.5 µg/mL. Mix well.
9. Pour the gel into a tray and insert the well comb, leave the gel to solidify for 10-15 mins at room temperature.
10. Once solidified, gel is ready for use.

Exolysin activity assay on double layer agar

1. Preparation of bottom LB agar
    - Prepare the LB medium according to the manufacturer's instructions, adding agar to a final concentration of 10 g/L and heating it to sterilize.
    - When the medium has cooled down to 55–60 °C, dispense 20 mL of medium per plate, and when cool, bag and store the plates at 4 °C.

- Bagged plates can be stored for up to 2 months.

2. Preparation of top agar
   - Prepare the LB medium according to the manufacturer's instructions, with the addition of agar to a concentration of 5 g/L and heating it to sterilize.
   - Before the experiment, heat the agar to melt it.
   - Using a sterile pipette, promptly dispense 3 mL volumes of the medium into sterile 13 × 100 mm glass tubes, cap the tubes with metal or plastic caps and store at 55-60 °C for no more than 8 hours.
3. Exolysin activity assay
   - Using a sterile loop, pick up one colony of *K. pneumoniae* and mix it into 5 mL of LB medium in a 100 mL flask.
   - Grow overnight at 30 °C, 180 RPM.
   - Next day make a 1:50–1:100 dilution of the overnight inoculate and incubate it in a shaking water bath at 30 °C, 180 RPM until OD_{600} reaches 0.4.
   - In the meantime, prepare bottom and top agars (protocol above).
   - Mix 500 µL of OD_{600} = 0.4 *K. pneumoniae* into 3 mL of top agar. After mixing, quickly pour it onto the bottom agar. Allow the top agar to dry for 10-15 minutes.
   - Afterwards, add 5 µL of exolysin. Allow the drop to seep into the top agar until there is no visible droplet left. Place the petri dish into a 30 °C thermostat for 12-16 hours.
   - Observe the plaques the next day.

PCR

PCR - Phusion DNA Polymerase

This protocol has been taken from Thermo Scientific website and is as follows:

Pipette these reagents in the order listed:

Component	**20 µL rxn**	**50 µL rxn**	**Final conc.**
H_2O	Add to 20 µL	Add to 50 µL	
5X Phusion HF	4 µL	10 µL	1X
10 mM dNTPs	0.4 µL	1 µL	200 µM each
Forward primer**	X µL	X µL	0.5 µM
Reverse primer**	X µL	X µL	0.5 µM
(DMSO***, optional)	(0.6 µL)	(1.5 µL)	(3%)
Phusion DNA polymerase	0.2 µL	0.5 µL	0.02 U/µL

*Optionally 5X Phusion GC Buffer can be used.

**Recommended final primer concentration is 0.5 µM, however it can vary in a range of 0.2-1.0 µM, if needed.

*** Addition of DMSO is recommended for GC-rich amplicons. DMSO is not recom-

mended for amplicons with very low GC % or amplicons that are > 20 kb.

PCR cycling program:

Cycle step	2-step protocol		3-step protocol		Cycles
	Temperature	Time	Temperature	Time	
Initial denaturation	98 °C	30 s	98 °C	30 s	1
Denaturation	98 °C	5-10 s	98 °C	5-10 s	25-35
Annealing	-	-	X °C	10-30 s	
Extension	72 °C	15-30 s/kb	72 °C	15-30 s/kb	
Final Extension	72 °C 4 °C	5-10 min hold	72 °C 4 °C	5-10 min hold	1

Note: Initial denaturation temperature, annealing temperature, and extension time are both primer and polymerase dependent, thus should be adjusted accordingly.

PCR - Taq DNA Polymerase

This protocol has been taken from Thermo Scientific website and is as follows:

Pipette these reagents in the order listed:

Component	20 µL rxn	50 µL rxn	Final concentration / amount
DreamTaq PCR Master Mix (2X)	10 µL	25 µL	
Forward primer*	X µL	X µL	0.1-1 µM
Reverse primer*	X µL	X µL	0.1-1 µM
Template DNA**	X µL	X µL	10 pg - 1 µg
Water, nuclease-free	Up to 20 µL	Up to 50 µL	

*Recommended final primer concentration is 0.4 µM, however it can vary in a range of 0.1-1.0 µM, if needed.

**Optimal amounts of template DNA for a 50 µL reaction volume are 0.01-1 ng for both plasmid and phage DNA, and 0.1-1 µg for genomic DNA. Greater amounts of template increase the risk of generation of non-specific PCR products. Smaller amounts of template reduce the accuracy of the amplification.

PCR cycling program:

Cycle step	3-step protocol		Cycles
	Temperature	Time	
Initial denaturation	95 °C	1-3 min	1
Denaturation	95 °C	30 s	25-40
Annealing	55 (T_m-5) °C	30 s	
Extension	72 °C	60 s/kb	
Final Extension	72 °C 4 °C	5-15 min hold	1

Note: Initial denaturation temperature, annealing temperature, and extension time are both primer and polymerase dependent, thus should be adjusted accordingly.

PCR - SuperFi DNA Polymerase

This protocol has been taken from Thermo Scientific website and is as follows:

Pipette these reagents in the order listed:

Component	**20 µL rxn**	**50 µL rxn**	**Final concentration / amount**
2X Platinum™ SuperFi™ PCR Master Mix	10 µL	25 µL	
Forward primer	X µL	X µL	0.5 µM
Reverse primer	X µL	X µL	0.5 µM
Template DNA	X µL	X µL	varies*
Water, nuclease-free	Up to 20 µL	Up to 50 µL	

*5–50 ng gDNA or 1 pg–10 ng plasmid DNA (see Optimization strategies for more information).

PCR cycling program:

Cycle step	**2-step protocol (<10kb)**		**3-step protocol (<10kb)**		**Long PCR (>10kb)**		**Cycles**
	Temperature	**Time**	**Temperature**	**Time**	**Temperature**	**Time**	
Initial denaturation	98 °C	30 s	98 °C	30 s	95 °C	2 min	1
Denaturation	98 °C	5-10 s	98 °C	5-10 s	95 °C	10 s	25-35
Annealing	-	-	X °C	10 s	X °C	10 s	
Extension	72 °C	15-30 s/kb	72 °C	15-30 s/kb	68 °C	30 s/kb	
Final Extension	72 °C 4 °C	5 min hold	72 °C 4 °C	5 min hold	68 °C 4 °C	5 min hold	1

Note: Initial denaturation temperature, annealing temperature, and extension time are both primer and polymerase dependent, thus should be adjusted accordingly.

PCR - Asymmetric Taq

This protocol has been taken from Thermo Scientific website and adapted as follows:

Pipette these reagents in the order listed:

**Optimal amounts of template DNA for a 50 µL reaction volume are 0.01-1 ng for both plasmid and phage DNA, and 0.1-1 µg for genomic DNA. Greater amounts of template in-

Component	20 µL rxn	50 µL rxn	Final concentration / amount
DreamTaq PCR Master Mix (2X)	10 µL	25 µL	
Forward primer	X µL	X µL	70 nM
Reverse primer	X µL	X µL	1050 nM
Template DNA**	X µL	X µL	10 pg - 1 µg
Water, nuclease-free	Up to 20 µL	Up to 50 µL	

crease the risk of generation of non-specific PCR products. Smaller amounts of template reduce the accuracy of the amplification.

PCR cycling program:

Cycle step	3-step protocol		Cycles
	Temperature	Time	
Initial denaturation	95 °C	1-3 min	1
Denaturation	94 °C	30 s	25-40
Annealing	57 (T_m-5) °C	30 s	
Extension	72 °C	60 s/kb	
Final Extension	72 °C 4 °C	5-15 min hold	1

Note: Initial denaturation temperature, annealing temperature, and extension time are both primer and polymerase dependent, thus should be adjusted accordingly.

PCR - Asymmetric SuperFi

This protocol has been taken from Thermo Scientific website and is as follows:

Pipette these reagents in the order listed:

Component	20 µL rxn	50 µL rxn	Final concentration / amount
2X Platinum™ SuperFi™ PCR Master Mix	10 µL	25 µL	
Forward primer	X µL	X µL	70 nM
Reverse primer	X µL	X µL	1050 nM
Template DNA*	X µL	X µL	varies*
Water, nuclease-free	Up to 20 µL	Up to 50 µL	

*5–50 ng gDNA or 1 pg–10 ng plasmid DNA (see Optimization strategies for more information).

PCR cycling program:

Cycle step	2-step protocol (<10kb)		3-step protocol (<10kb)		Long PCR (>10kb)		Cycles
	Temperature	Time	Temperature	Time	Temperature	Time	
Initial denaturation	98 °C	30 s	98 °C	30 s	95 °C	2 min	1
Denaturation	98 °C	5-10 s	98 °C	5-10 s	95 °C	10 s	25-35
Annealing	-	-	X °C	10 s	X °C	10 s	
Extension	72 °C	15-30 s/kb	72 °C	15-30 s/kb	68 °C	30 s/kb	
Final Extension	72 °C 4 °C	5 min hold	72 °C 4 °C	5 min hold	68 °C 4 °C	5 min hold	1

Note: Initial denaturation temperature, annealing temperature, and extension time are both primer and polymerase dependent, thus should be adjusted accordingly.

Colony PCR

1. Prepare a PCR Master Mix as per the previously noted PCR protocols.
    - Note: Due to pipetting losses make sure to prepare a 10% greater volume of PCR Master Mix.
2. Prepare final primer concentrations of 0.4 µM and run the PCR for 35 cycles.
3. Transfer the Master Mix in 20 µL or 50 µL quantities to small PCR tubes.
4. Take the dish with bacterial colonies intended for testing.
5. Using a sterile instrument, transfer an individual colony to a separate dish marking the transferred colonies. Then, use the same instrument and shake the tip of it inside the aliquot of the Master Mix.
6. Repeat for a desired number of colonies.
7. Incubate the second dish at the optimal temperature for bacterial growth.
8. Load the PCR tubes into the PCR thermocycler and run the adequate program.
    - Note: If the results prove to be successful, colonies from the second dish can be used for further experiments as the bacteria usually proliferate enough to be transferred into liquid media for overnight growth.

Annealing oligos for lengthier primers

Protocol provided by Addgene

1. Place the mixed oligos in a 1.5 mL microfuge tube.
2. Place the tube in a 90-95 °C dry bath and incubate for 3-5 minutes.
3. Gradually cool down to 25 °C over 45 minutes.

Gel electrophoresis

1. Remove the comb from the cast gel.
2. Place the gel into an electrophoresis system and make sure that the volume of the buffer is sufficient.
3. Add the DNA size marker or ladder (3-5 µL).
4. After submerging the wells of the gel, load the samples.
5. Load 8-50 µL of the sample depending on the purpose of the gel.
6. Set voltage to 120 V.
7. Set the appropriate gel run time.
    - Note: 20 minutes are sufficient for a gel with already incorporated ethidium bromide, however the time can be longer or shorter depending on the length of the DNA fragments.
8. Place the electrodes with the cover over the gel system and start the electrophoresis.
9. After the run is over, switch off the system.
10. Remove the gel and place it over a UV or Blue-light illuminator to visualize the EtBr intercalated DNA either for confirmation of experimental results or gel excision and DNA purification.

Gel extraction

1. Remove the gel from the electrophoresis system.
2. To visualize the DNA prior to extraction one cannot use UV illuminators due to risk of mutation caused by UV exposure. Instead, a Blue-light illuminator in a dark room can be used, which is sufficient for accurate gel excision.
    - Note: If the possibility of avoiding UV irradiation is out of the question, please try to keep the exposure of the gel to UV under 10 seconds to avoid damaging DNA.
3. Excise the bands with the DNA of interest keeping the excess of gel to a minimum as it decreases the yield of DNA after cleanup.
4. Follow the Thermo Scientific GeneJET Gel Extraction and DNA Cleanup Micro Kit, which is listed below as follows:
5. Excise a gel slice of up to 200 mg containing the DNA fragment using a clean scalpel or razor blade. Cut as close to the DNA as possible to minimize gel surplus. Place the gel slice into a 1.5 mL tube.
6. Add 200 µL of Extraction Buffer. Mix thoroughly by pipetting.
7. Incubate the gel mixture at 50-58 °C for 10 minutes or until the gel slice is completely dissolved. Mix the tube by inversion every few minutes to facilitate the melting process. Ensure that the gel is completely dissolved.
8. Add 200 µL of ethanol (96-100%) and mix by pipetting.
9. Transfer the mixture to the DNA Purification Micro Column preassembled with a

collection tube. Centrifuge the column for 30-60 seconds at 14,000 RCF. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube.

- Note:
  - If DNA fragment is ≥ 10 kb centrifuge the column for 2 minutes at 14,000 RCF.
  - Close the bag with DNA Purification Micro Columns tightly after each use!

10. Add 200 µL of Prewash Buffer (with ethanol) to the DNA Purification Micro Column and centrifuge for 30-60 seconds at 14,000 RCF. Discard the flow-through and place the purification column back into the collection tube.
    - Note. If DNA fragment is ≥ 10 kb centrifuge the column for 1-2 minutes at 14,000 RCF.
11. Add 700 µL of Wash Buffer (with ethanol) to the DNA Purification Micro Column and centrifuge for 30-60 seconds at 14,000 RCF. Discard the flow-through and place the purification column back into the collection tube.
    - Note. If DNA fragment is ≥ 10 kb centrifuge the column for 1-2 minutes at 14,000 RCF.
12. Repeat step 11.
13. Centrifuge the empty DNA Purification Micro Column for an additional 1 minute at 14,000 RCF to completely remove the residual Wash Buffer.
    - Note. This step is essential to avoid residual ethanol in the purified DNA solution. The presence of ethanol in the DNA sample may inhibit downstream enzymatic reactions.
14. Transfer the DNA Purification Micro Column into a clean 1.5 mL microcentrifuge tube (not included).
15. Add 10 µL of Elution Buffer to the DNA Purification Micro Column. Centrifuge for 1 minute at 14,000 RCF to elute DNA.
    - Note:
      - If a DNA fragment is ≥ 10 kb the elution volume should be increased between 15-20 µL to get optimal DNA yield. Elution volume less than 10 µL is not recommended.
      - Lower volume of Elution Buffer for DNA Micro Kit can be used (6-10 µL) in order to concentrate eluted DNA. Please notice that <10 µL elution volume slightly decreases DNA yield.
16. Discard the purification column and store the purified DNA at -20 °C.

Enzymatic reaction cleanup

This protocol was provided by Thermo Scientific and is as follows:

1. Adjust the volume of the reaction mixture to 200 µL with Water, nuclease-free or TE buffer (not included).
2. Add 100 µL of Binding Buffer. Mix thoroughly by pipetting.
3. Add 300 µL of ethanol (96-100%) and mix by pipetting.
4. Transfer the mixture to the DNA Purification Micro Column preassembled with a collection tube. Centrifuge the column for 30-60 seconds at 14,000 RCF. Discard the flow-through. Place the DNA Purification Micro Column back into the collection tube.
    - Note:
        - If DNA fragment is ≥ 10 kb centrifuge the column for 2 minutes at 14,000 RCF.
        - Close the bag with DNA Purification Micro Columns tightly after each use!
5. Add 700 µL of Wash Buffer (with ethanol) to the DNA Purification Micro Column and centrifuge for 30-60 seconds at 14,000 RCF. Discard the flow-through and place the purification column back into the collection tube.
    - Note. If DNA fragment is ≥ 10 kb centrifuge the column for 2 minutes at 14,000 RCF.
6. Repeat step 5.
7. Centrifuge the empty DNA Purification Micro Column for an additional 1 minute at 14,000 RCF to completely remove residual Wash Buffer.
    - Note. This step is essential to avoid residual ethanol in the purified DNA solution. The presence of ethanol in the DNA sample may inhibit downstream enzymatic reactions.
8. Transfer the DNA Purification Micro Column into a clean 1.5 mL microcentrifuge tube (not included).
9. Add 10 µL of Elution Buffer to the center of the DNA Purification Micro Column membrane. Centrifuge for 1 minute at 14,000 RCF to elute DNA.
    - Note:
        - If a DNA fragment is ≥ 10 kb the elution volume should be increased to 15-20 µL to achieve optimal DNA yield.
        - Lower volume of Elution Buffer for DNA Micro Kit can be used (6-10 µL) in order to concentrate eluted DNA. Please notice that < 10 µL elution volume slightly decreases DNA yield.
        - Double the elution volume or perform two elution cycles when purifying larger amounts of DNA (for example > 5 µg).
10. Discard the purification column and store the purified DNA at -20 °C.

DNA digestion

This protocol is taken from Thermo Fisher Scientific website and is as follows:

1. Prepare the reaction mixture at room temperature in the order indicated.

Component	**Volume**		
	Plasmid DNA	**Unpurified PCR product**	**Genomic DNA**
Water, nuclease-free*	15 µL	17 µL	30 µL
10X FastDigest buffer or 10X FastDigest Green buffer	2 µL	2 µL**	5 µL
DNA	2 µL (up to 1 µg)	10 µL(~0.2 µg)	10 µL (5 µg)
FastDigest enzyme	1 µL	1 µL	5 µL
Total volume	**20 µL**	**50 µL**	**50 µL**

2. Mix gently and spin down.
3. Incubate at 37 °C in a heat block or water thermostat for 5 min. ***
4. Inactivate the enzyme(s) (optional). ***
    - Note:
        - *The volume of water should be corrected to keep the indicated total reaction volume. The volume of DNA can be scaled up to 10 µL or down to 0.5 µL depending on the DNA concentration.
        - **Only 2 µL of 10X FastDigest® buffer is required for unpurified PCR product in a 30 µL reaction volume.
        - ***See the Certificate of Analysis for enzyme and substrate specific incubation time and enzyme inactivation conditions.

Ligation

This protocol is taken from Thermo Scientific website and is as follows:

1. Thoroughly mix the 5X Rapid Ligation buffer prior to use.
2. Add the following to a microcentrifuge tube:

Linearized vector DNA	10-100 ng
Insert DNA (at 3:1 molar excess over vector)	variable
5X Rapid Ligation Buffer	4 µL
T4 DNA Ligase, 5 U/µL	1 µL
Water, nuclease-free	To 20 µL
Total volume	**20 µL**

3. Vortex and spin briefly to collect the drops
4. Incubate the mixture at 22 °C for 5 min.
5. Use 2-5 µL of the ligation mixture for transformation.

Bacteria lysis with ultrasound

1. After recombinant protein synthesis induction, collected biomass is weighed and resuspended with either Tris-HCL (pH = 8) or PBS (pH = 7.2) buffer and transferred to the ice bath.
2. Bacteria are lysed with ultrasound for 2 minutes by sending short 2-3 s 750 W impulses each 20 seconds by using VC-750 ultrasound processor (Sonics Materials, Inc.).
3. Cell debris is discarded by centrifuging the sample for 20 minutes at 16000 RCF speed at 4 °C.
4. The supernatant is further used in protein purification.

Protein Purification

1. *E. coli* lysates with an appropriate induced protein are purified using a metal chelating Ni-NTA column (GE Healthcare), which is used for recombinant proteins with 6xHis tag.
   (a) Proteins with maltose binding protein tag were purified with MBPTrap™ HP column (GE Healthcare), which is pre-packed with Dextrin Sepharose™.
   (b) Proteins with Strep-tag™ II were purified using StrepTrap™ HP column (GE Healthcare), which is pre-packed with StrepTactin Sepharose™.
2. Appropriate columns were equilibrated with 5 CV (column volumes) of binding buffer (50 mM Tris-HCl, 300 mM NaCl, pH = 8).
3. Then the protein sample was injected onto the column.
4. Afterwards, the column was washed with 5 to 10 CV of binding buffer or until no material appeared in the effluent.
5. Finally, desired protein was eluted with 8 CV of elution buffer.
   (a) Proteins with 6xHis tag were eluted with a binding buffer containing 0.5 M imidazole.
   (b) Proteins with maltose binding protein were eluted with a binding buffer containing 10 mM maltose.
   (c) Proteins with *Strep*-tag II were eluted with a 2.5 mM desthiobiotin binding buffer.

Protein dialysis

After protein purification there is unwanted imidazole left which could interfere with our further experiments. 12 kDa semi-permeable dialysis tube (Carl Roth) was used for all dialysis.

1. Firstly, the dialysis tube is soaked in distilled water, one of its ends is clipped with a clipper and protein solution is loaded into the membrane without leaving any air

bubbles and then the upper end of the membrane is clipped.
2. Dialysis is performed at 4 °C overnight in one liter of the appropriate binding buffer (either PBS, NaCl 0.3 M pH 7.4 or Tris-HCl 50 mM, NaCl 0.3 M pH8).
3. After dialysis, to concentrate the protein, the dialysis tube is sprinkled with sodium carboxymethylcellulose powder. It absorbs water through the semipermeable membrane. Sufficient amount of time was allowed for the protein solution to reach desirable volume.
4. Afterwards, the protein concentration is measured using NanoDrop 2000 Spectrophotometer (Thermo Scientific).

Protein Electrophoresis (SDS Page)

Separating gel

1. Prepare the gel casts in a container. Fill the container with water and after 5 min check for leakage. After a leakage check, remove water from the gel casts and dry residual gel with a paper towel.
2. To prepare a separation-gel, mix the reagents listed in the table below in proper volumes to produce a gel of desired composition.
    - NOTE! Add TEMED and APS last, as solidification of the gel will occur when these reagents are added.
3. Pipette gel mixture into gel casts up to a height approximately 1 cm below the gel's comb.
4. Fill the remaining cast with isopropanol (if not available use distilled water).
5. Allow the gel to harden for 30-60 min.
6. Pour of all the isopropanol (or water) from gel casts and dry the gel with paper towels.
    - Ammonium Persulfate (APS) - 20 mg/mL
    - Acrylamide 30% solution
    - TEMED (99.9%)
    - Separating Gel Buffer - 1.5 M Tris-HCl pH 8.8 with 0.4% SDS. Stored at 4 °C.
    - Stacking Gel Buffer - 0.5 M Tris-HCl pH 6.8 with 0.4% SDS. Stored at 4 °C

1 Table.: Percentage of Acrylamide, depending on protein size.

Acrylamide [%]	**M. W. Range [kDa]**
7	50 - 500
10	20 - 300
12	10 - 200
15	3 - 100

2 Table.: Separating gel composition

Acrylamide [%]	**6% [mL]**	**8% [mL]**	**10% [mL]**	**12% [mL]**	**15% [mL]**
H_2O	5.2	4.6	3.8	3.2	2.2
Acrylamide/Bis-acrylamide (30% / 0.8%, w/v)	2	2.6	3.4	4	5
1.5 M Tris, pH 8.8	2.6	2.6	2.6	2.6	2.6
10% (w/v) SDS	0.1	0.1	0.1	0.1	0.1
10% (w/v) ammonium persulfate (APS)	0.1	0.1	0.1	0.1	0.1
TEMED	0.01	0.01	0.01	0.01	0.01

Stacking gel

1. To prepare a stacking-gel, in a tube, mix the reagents listed in table 3 below with the proper volumes to receive a gel with desired composition.
    - NOTE! Add TEMED and APS last, as solidification of the gel will occur when these reagents are added.
2. Pipette gel mixture on top of a separating gel to fill the cast and remove all air bubbles.
3. Fill the remaining cast with isopropanol (if not available use distilled water).
4. Insert the comb whilst the gel is still in liquid form. The comb will form wells to load samples into when the gel turns solid.
5. Allow to harden for 45-60 min.

3 Table.: Stacking gel composition

Material	**Amount [mL]**
H_2O	2.975
0.5 M Tris-HCl, pH 6.8	1.25
10% (w/v) SDS	0.05
Acrylamide/Bis-acrylamide (30%/0.2% w/v)	0.67
10% (w/v) ammonium persulfate (APS)	0.05
TEMED	0.005

Running the gel

1. Mount the gels into the tank, remove combs and top off the inner chamber of the tank with 1X running buffer. Fill a third of the outer chamber of the tank with 1X running buffer.
2. Add 2 µL of ladder and 10 µL of sample into each well.
3. Connect the power supply and run at 100 V for 60 min or until the blue line approaches the bottom of the gel.
4. Once electrophoresis is done, remove the gels from the glass plates.
5. Carefully wash all equipment used for electrophoresis with water.

10x Running buffer

Dissolve 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 mL of H_2O. The pH buffer should be 8.3 and no pH adjustment is required. Store the running buffer at room temperature and dilute to 1X before use.

Coomassie Blue Staining

1. Prepare the staining solution containing 0.1% Coomassie Blue in 40% ethanol, 10% acetic acid.
2. After electrophoresis, incubate 1 or 2 gels in a staining container containing 100 mL of Coomassie Blue staining solution.
3. Incubate at room temperature for 1 hour until bands are visible.
4. Decant the stain and rinse the gel once with deionized water.
5. Prepare 100 mL of destaining solution containing 10% ethanol and 7.5% acetic acid.
6. Gently shake the gel at room temperature on an orbital shaker until the desired background is achieved.

Biofilm formation

1. In order to obtain a biofilm, one colony of *Klebsiella pneumoniae* is transferred to a 5 mL LB miniprep and grown for 24h at 37 °C, 180 RPM.
2. Then, 0.5 mL of overnight culture is transferred to the fresh 10 mL of LB medium in a glass container (flask or tube) and is grown for 48 hours without shaking.
3. The LB medium is decanted or pippeted and the biofilm is left at the bottom of the glass container.

Alginate beads formation with GFP

Materials needed: 2.5% alginate solution (alginic acid dissolved in water (2.5 g/100 g distilled water), 0.1 M $CaCl_2$, and purified GFP.

1. Mix alginate solution and GFP at the ratio of 4:1.
2. Load the mixture into the syringe with a needle.
3. While holding the syringe above the container with $CaCl_2$ slowly release droplets of mixture into the $CaCl_2$ solution.

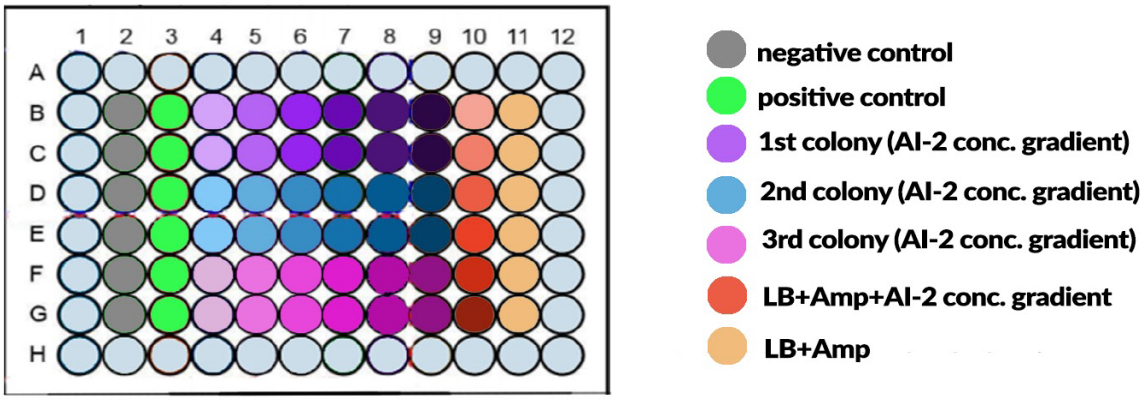
Fluorescence measurement using a plate reader

Introduction: This protocol is for measurement of fluorescence and OD_{600}.

Procedure

Preparation of bacteria

1. Transform bacteria with plasmid containing your target genes.
2. Pick three colonies from each plate and inoculate in 5 mL of LB medium with the right antibiotic.
3. Make a 1:10 dilution of each overnight culture in LB medium with the right antibiotic.
4. Measure OD_{600} and write down the values of OD_{600}.
5. Dilute the culture to 0.05 OD_{600} in an eppendorf tube. The sample is placed on ice.
6. The samples should be laid out according to the plate down below



WESTERN BLOT

Solutions

30% acrylamide – bis solution

30 g acrylamide and 0.8 g N,N'-methylenebisacrylamide are dissolved in 100 mL of deionized H_2O. The solution is filtered and stored at 4 °C, no longer than 30 days.

1.5 mol/l Tris-HCl, pH=8.8

18.15 g Tris dissolved in 70 mL of H_2O; pH is corrected with HCl. Solution is diluted to 100 mL, filtered and stored at 4 °C, in the dark for no longer than 30 days.

0.5 mol/l Tris-HCl, pH=6.8

3 g of Tris are dissolved in 35 mL of H_2O, pH is adjusted using HCl. The solution is diluted to 50 mL, filtered and stored at 4 °C, in the dark for no longer than 30 days.

10% SDS solution

5 g of SDS are dissolved and diluted to 50 mL. The solution is stored at room temperature.

10% APS (ammonium peroxide sulphate)

25 mg of APS are dissolved in 250 µL of H_2O. Solution must be used on the day of preparation.

5X “Loading” buffer solution

Each of these components:

- 350 µL of 0.5 mol/L Tris-HCl (pH=6.8),
- 150 mL of glycerol,
- 50 mg of SDS,
- 46 mg of DTT,
- 10 µL of bromophenol blue,

are to be dissolved in H_2O one after the other. The solution is then frozen. Protein samples are heated up once mixed with this buffer, and before loading into the gel – cooled down.

10X PBS (phosphate buffer solution) (1 liter)

- 80.06 g of NaCl;
- 2.01 g of KCl;
- 11.4 g of Na_2HPO_4 (Na_2HPO_4*$7H_2O$ – 21.6 g; Na_2HPO_4*$12H_2O$ – 29.0 g);
- 2.04 g of KH_2PO_4.

Dissolve in 800 mL of deionized H_2O in the order that the components are listed. Dilute to 1 L. Pipette in 100 mL portions and store at 4 °C.

Protein transfer buffer solution

For 100 mL: 300 mg Tris, 1.12 g glycine, 10 mL methanol. Dilute with H_2O to 100 mL. (400 mL is the amount to be made).

Blocking solution

PBS with 2% milk powder.

Tween dilution

Dilute 5 mL of concentrated Tween to 50 mL. 1 L PBST 10 mL diluted Tween (Tween 10%/10x).

PBS-T

PBS + 0.1% Tween-20.

Chloronaphthol solution

1 tablet of chloronaphthol + 10 mL of methanol. For membrane resolution use 10 mL of PBS 2 mL chloronaphthol dissolved in methanol 30 µL 30% H_2O_2.

ELECTROPHORESIS - IMMUNOBLOTTING

Electrophoresis

1. Everything done in this step is covered in the SDS-PAGE protocol.
2. The protein from the polyacrylamide gel is transferred onto the PVDF membrane (Polyvinylidene fluoride). The membrane is cut to be nearly the same size as the gel, same sized filter papers are also prepared.
3. After electrophoresis, the polyacrylamide gel is submerged in the protein transfer solution. The membrane is washed with methanol and also soaked in the transfer solution. The same is done to the filter papers.
4. Semi-dry fractionated protein transfer onto the membrane is performed. On the protein transfer apparatus' cathode base a piece of soaked filter paper is placed, afterwards the membrane, gel, and lastly, another layer of filter paper. Everything is pressed down using the anode base and for 40 minutes the protein transfer is performed, the current is adjusted based on the area of the gel. (Length cm x width cm = area cm^2 mA usually 40 mA) (always check if there is an already set protein transfer current based on the lab you are working in).
5. The membrane is washed with PBST and afterwards blocked with PBS + 2% milk powder solution for 60 minutes at room temperature (can be stored at 4 °C overnight).
6. The membrane is washed 4 times (for 5 min) with PBST. The antibody solution is prepared: PBST + 2% milk powder. If the hybridoma medium is used, it can be diluted to 1:1-1:20 overall solution volume is 10 mL [50 µL hybridoma medium]. The membrane is incubated with the antibodies for 1 h at room temperature.
7. Membrane is washed 6 times with PBST. The secondary antibody solution is prepared with 2% milk powder PBST. The conjugate is diluted to 1:1000 – 1:5000 (usually. 1:4000), based on the manufacturer's recommendations. Incubate for 1 h at room temperature.
8. Membrane is washed 4 times with PBST, incubating 2–3 times for 5 min on a shaking platform. Afterwards, wash 2–3 times with distilled water.
9. The membrane is treated with chloronaphthol solution, and only if nothing is seen or is barely resolved, TMB – blotting substrate for the peroxidase (ready-to-use) is used (before TMB wash the membrane with water).
10. The membrane is washed with water, dried, and scanned. Due to light exposure the peroxidase substrate blurs out in 3-7 days.

Gold nanoparticles synthesis

1. Prepare two solutions: A and B.
   - **Solution A:** 80 mL of distilled water mixed with 1 mL, 1% $HAuCl_4$.

- **Solution B:** 4 mL of 1% tri-sodium citrate mixed with 16 mL of distilled water and 25 µL 1% tannic acid.

2. Both solutions were heated to 60 °C then solution A was added to solution B.
3. The resulting solution was heated to 95 °C and left for 5 min. Solution was left to cool down at room temperature.
4. For long term storage, the solution was kept at 4 °C.
5. The Malvern Panalytical NanoSight instrument which utilizes Nanoparticle tracking analysis (NTA) was used to characterize gold nanoparticles.

Detection probe preparation

1. 40 µL of 100 µM thiolated probe was mixed with 40 µL of 50 mM Phosphate buffer (PB buffer) and 4 µL (100 mM) DTT.
2. Mixture was incubated for 60 minutes at room temperature.
3. DTT extraction was made with ethyl acetate. 100 µL of ethyl acetate was added to the solution and after gentle pipetting the top layer was discarded and the procedure was repeated 4 times.
4. 40 µL of activated probe was mixed with 1 mL of Au-NP solution, 10 µL (1%) SDS and 20 µL (0.5 mM) PB buffer.
5. Mixture was incubated for 20 minutes at room temperature with 600 RPM.
6. After incubation every 30 minutes 2M NaCl was added to the solution until final concentration of 0.4 M. (50 µL at a time).
7. Solution was left to incubate overnight.
8. Finally, the solution was centrifuged for 25 minutes at 12 000 RPM at room temperature. After centrifugation, pellet was resuspended in 1 mL (0.1%) Tween20. Procedure was repeated three times. The final resuspension was made in 1 mL nuclease free water.
9. Functionalized Au-NP are kept in the fridge until further use.

Detection probe preparation (low pH method)

This method works best if probe DNA sequence has a poly-A end.

1. 45 µL of thiolated probe was mixed with 1 mL of Au-NP solution and left to incubate for 60 min.
2. After incubation, 500 mM sodium citrate, pH 3.0 stock solution was added until final 10 mM concentration.
3. Three minutes later, the sample was centrifuged (25 min, 12 000 RPM) to remove the nonconjugated free DNA and then redispersed in a MilliQ water.
4. For lateral flow assay experiments gold nanoparticles buffer can be resuspended in

buffer: 20 mM Na_3PO_4, 0.2% Tween20, 10% sucrose, 5 mg/mL BSA.

Capture and control probes preparation

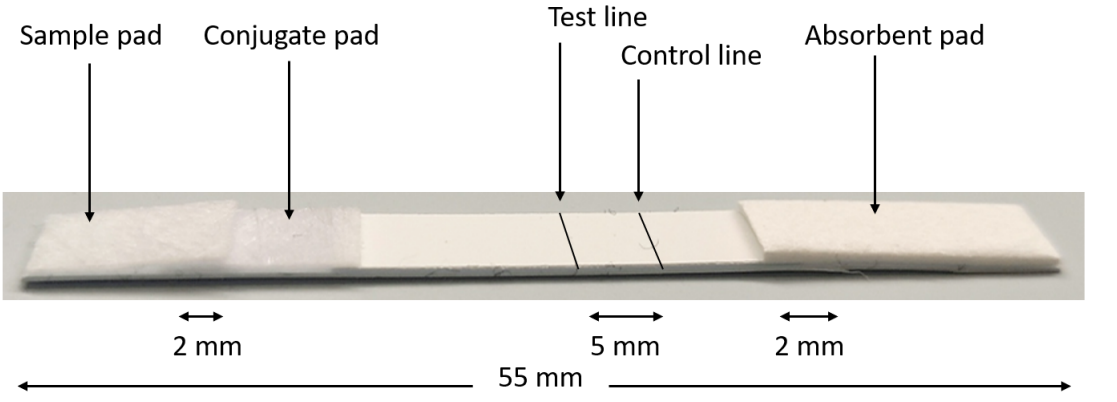
Both probes are prepared in the same way.

1. 91.7 µL of 100 µM biotinylated probe was mixed with 100 µL of 1 mg/mL streptavidin or avidin and 100 µL of 50 mM PB buffer.
2. Solution was incubated for 60 minutes at room temperature.

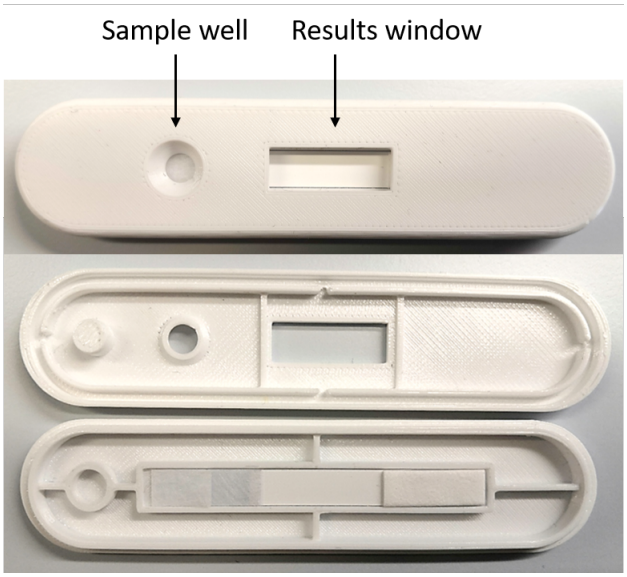
Lateral flow assay test preparation

1. On nitrocellulose membrane (HF180MC100) test and control lines are dispensed at a flow rate of 0.75 µL/cm using BioDot XYZ3060 dispense platform with BioJet dispenser.
2. Control probes are located on the control line and capture probes are located on the test line. Space between control and test probe was 5 mm.
3. Functionalized Au-NP are dispensed on the conjugate pad (GFCP10300) at a flow rate of 25 µL/cm using BioDot XYZ3060 dispense platform with AirJet dispenser.
4. Finally, absorbent pad (CFSP173000), sample pad (GFCP10300) and conjugate pad are glued onto the nitrocellulose backing card (HF180MC100) with 2 mm overlaps.
5. Assembled lateral flow assay membrane sheet was cut into 6 mm width strips by BioDot CM5000 guillotine cutter.

1 Figure: Lateral flow assay test parameters.



2 Figure: Case designed by Micromolds for lateral flow assay test.



Lateral flow assay

1. Desired amount of ssDNA amplicon is applied to the sample pad. If wanted, sample pad can be pretreated with a buffer: 0.05 M Tris-HCl, 0.25% Triton X-100, 0.15 M NaCl.
2. After sample addition, 100 µL of running buffer is applied on the sample pad. As an alternative the test strip can be immersed in 200 µL of running buffer.
3. As running buffers we tried:
    - MilliQ ultra clean water
    - 50 mM phosphate buffer
    - Running buffer I (8X SSC, 1% Tween20, 1% SDS)
    - Running buffer II (10X SSC, 3.5% Triton X-100, 0.25% SDS, 12.5% Formamide).
4. Results were photographed after 15 - 25 minutes.

Genomic DNA extraction

Protocol adapted from GeneJET Genomic DNA Purification Kit protocol.

1. Harvest up to $2x10^9$ bacterial cells in a 1.5 or 2 mL microcentrifuge tube by centrifugation for 10 min at 5000 RCF. Discard the supernatant.
2. Resuspend the pellet in 180 µL of Digestion Solution. Add 20 µL of Proteinase K Solution and mix thoroughly by vortexing or pipetting to obtain a uniform suspension.
3. Incubate the sample at 56 °C while vortexing occasionally in the thermomixer until the cells are completely lysed (30 min).
4. Add 20 µL of RNase A Solution, mix by vortexing and incubate the mixture for 10 min at room temperature.

5. Add 200 µL of Lysis Solution to the sample. Mix thoroughly by vortexing for about 15 s until a homogeneous mixture is obtained.
6. Add 400 µL of 50 ethanol and mix by pipetting or vortexing.
7. Transfer the prepared lysate to a GeneJET Genomic DNA Purification Column inserted in a collection tube. Centrifuge the column for 1 min at 6000 RCF. Discard the collection tube containing the flow-through solution. Place the GeneJET Genomic DNA Purification Column into a new 2 mL collection tube.
8. Add 500 µL of Wash Buffer I. Centrifuge for 1 min at 8000 RCF.
9. Discard the flow-through and place the purification column back into the collection tube.
10. Add 500 µL of Wash Buffer II to the GeneJET Genomic DNA Purification Column. Centrifuge for 3 min at maximum speed (≥12000 RCF). Discard the collection tube containing the flow-through solution and transfer the GeneJET Genomic DNA Purification Column to a sterile 1.5 mL microcentrifuge tube.
11. Add 200 µL of Elution Buffer to the center of the GeneJET Genomic DNA Purification Column membrane to elute genomic DNA. Incubate for 25 min at room temperature and centrifuge for 1 min at 8000 RCF.
12. Discard the purification column. Use the purified DNA immediately in downstream applications (PCR) or store at -20 °C.

Primer design and amplicon selection for HDA

tHDA primers can be designed using the IDT Primerquest tool or Primer3 program.
Recommended parameter settings:

1. Product size: 80 - 120 bp
2. Product T_m: Min 68: Opt 71: Max 75 °C
3. Primer size: Min 24: Opt 27: Max 33
4. Primer T_m: Min 60: Opt 68: Max 74 °C
5. Primer GC%: Min 35: Opt 44: Max 60

One-Step tHDA (thermostable HDA)

Protocol adapted from New England Biolabs website.

1. Prepare a 50 µL reaction in a 0.2 mL or a 0.5 mL microcentrifuge tube in a sterile hood or a PCR Workstation.
2. Mix the solution by vortexing or by pipetting, followed by a short centrifugation. Cover the reaction mixture with 50 µL of mineral oil. Place the tubes on ice.
3. Incubate at 65 °C for 90-180 minutes.
4. Load 15 µL of the tHDA product on a 2.5% TBE agarose gel.

	Symmetric HDA	Asymmetric HDA
H_2O	X µL	X µL
10X Annealing buffer II	5 µL	5 µL
$MgSO_4$ (100 mM)	2 µL	2 µL
NaCl (500 mM)	4 µL	4 µL
IsoAmp® dNTP Solution	3.5 µL	3.5 µL
DNA template*	100 - 200 ng	50 - 150 ng
Forward Primer (5 µM)	0.75 µL	100 nM
Reverse Primer (5 µM)	0.75 µL	300 nM
IsoAmp® Enzyme Mix	3.5 µL	3.5 µL
Total volume	**50 µL**	**50 µL**

*DNA template amount that worked the best in our experiments

The conditions of tHDA reactions can be further optimized:

Component	**Recommended concentration**
$MgSO_4$	3.5 to 4 mM
NaCl	30 to 40 mM
Primer	75 to 100 nM
DMSO	1 - 5%
Sorbitol	0.05 - 0.25 mol/L
Formamide	1.25 - 10%
Dextran	5 - 15%
Ficoll	5 - 15%
PEG	1 - 15%
Restriction prior to amplification might help in getting better amplicon yields	
Amplification temperature	55 to 75 °C
Amplification time	60 to 180 min

Two-Step tHDA (thermostable HDA)

Protocol adapted from New England Biolabs website.

1. To set up a 50 µL tHDA reaction, prepare a 25 µL Mix A in a 0.5 mL microcentrifuge tube in a sterile hood or a PCR Workstation:

	Symmetric HDA	Asymmetric HDA
H_2O	X µL	X µL
10X Annealing buffer II	2.5 µL	2.5 µL
DNA template*	100 - 200 ng	50 - 150 ng
Forward Primer (5 µM)	0.75 µL	100 nM
Reverse Primer (5 µM)	0.75 µL	300 nM
Total volume of Mix A	**25 µL**	**25 µL**

2. Additionally, prepare a 25 µL Mix B in a separate 0.5 mL microcentrifuge tube:

	Symmetric HDA	Asymmetric HDA
H_2O	9.5 µL	9.5 µL
10X Annealing buffer II	2.5 µL	2.5 µL
$MgSO_4$ (100 mM)	2 µL	2 µL
NaCl (500 mM)	4 µL	4 µL
IsoAmp® dNTP Solution	3.5 µL	3.5 µL
IsoAmp® Enzyme Mix	3.5 µL	3.5 µL
Total volume of Mix B	**25 µL**	**25 µL**

3. Gently mix each of the mixes by vortexing or by pipetting followed by a short centrifugation. Cover the reaction mixture with 50 µL of mineral oil. Place the tubes on ice.
4. Incubate Mix A at 95 °C for 2 minutes and place immediately on ice. Add 25 µL of Mix B into Mix A underneath the oil layer and gently mix the reaction by pipetting. Place the tubes on ice.
5. Incubate at 65 °C for 120 minutes.
6. Load 15 µL of the tHDA product on a 2.5% agarose gel.

*DNA template amount that worked the best in our experiments

Preparation of chemically competent yeast cells

1. Inoculate 3 mL of YEPD medium with a chosen yeast strain and incubate overnight (16-18 hrs) in a shaking water bath at 30 °C, 230-270 RPM.
2. Dilute the culture 10 times by adding 333 µL of overnight culture to 3 mL of fresh YEPD medium.
3. Incubate the diluted yeast for another 3 hrs in a shaking water bath at 30 °C, 230-270 RPM.
4. Centrifuge the cells for 5 min at 2000 RPM.
5. After centrifugation, remove the supernatant and add 1 mL of distilled water.
6. Centrifuge the cells for 5 min at 2000 RPM.
7. Once again remove the supernatant before adding 300 µL of 100 mM TE-LiAc solution and incubating for 1 hr at 30 °C.
8. After incubation, centrifuge the cells for 3 min at 3000 RPM.
9. Remove the supernatant, resuspend the cells with 100 µL of 100 mM TE-LiAc solution.

Transformation of chemically competent yeast cells

1. Pipette 80 µL of the previously prepared competent cell solution and add 10 µL of 96% ethanol as well as 5 µL of chloroform-extracted plasmid DNA (up to 1 µg of total

DNA).
2. Incubate for 30 min at room temperature.
3. Add 300 µL of 50% TE-PEG, 30 µL of 1 M LiOAc, suspend the solution and incubate for 1 hr at 30 °C.
4. Incubate for 20 min at 42 °C.
5. Centrifuge the cells for 3 min at 3000 RPM.
6. Remove supernatant and resuspend the cells with 1 mL of YEPD medium.
7. Centrifuge the cells for 3 min at 3000 RPM.
8. Remove supernatant and resuspend the cells with 0.5 mL of YEPD medium.
9. Incubate the cells overnight in sterile conditions at 30 °C and with the test-tube lid open (cover the opening with perforated foil instead).
10. Next day, inoculate the yeast onto plates with YEPD + formaldehyde (0.2 µL of formaldehyde per 1 mL of YEPD) (40 µL of 37% formaldehyde into 100 mL).
11. After another two days, inoculate the yeast into plates with YEPD + formaldehyde (0.4 µL of formaldehyde per 1 mL of YEPD) (60 µL of 37% formaldehyde into 100 mL).

Recombinant protein synthesis in yeast

1. Yeast, containing the desired protein gene, are incubated in a flask (medium taking up no more than 10% of its total volume) for 24-48 hrs.
2. Centrifuge the cells for 5 min at 3000 RPM and remove the supernatant.
3. Resuspend the cells with 6 mL of 2X YEP, 6 mL of 12% galactose solution and 3.6 µL of formaldehyde and transfer them into a 100 mL flask with a cloth stopper.
4. Incubate the cells for 16-24 hrs in a shaking water bath at 30 °C at 220 RPM.
5. Before induction, remove a 1 mL sample, centrifuge it for 1 min at 13 000 RPM and remove the supernatant.
6. Add 50 µL of 1:1 mixture (by volume) of 2X loading dye and 2-Mercaptoethanol and mix by pipetting.
7. Incubate the mixture at 100 °C for 5 min. Afterwards, freeze it at -20 °C until sample SDS-PAGE electrophoresis can be performed.
8. Steps 5 through 7 are to be repeated at 5, 16 and 24 hr time points after the start of induction.

Preparation of yeast cell lysate

All steps are to be carried out with the samples placed on ice.

1. After induction, transfer the entire mixture into a 50 mL Falcon tube.
2. Centrifuge the tube with its contents for 10 min at 3000 RPM at 4 °C.
3. Remove supernatant and wash the remaining biomass with 45 mL of distilled water.

4. Repeat step 2.
5. Remove the supernatant and weigh the remaining biomass.
6. Add lysis buffer (1:2 / biomass:lysis buffer by volume).
7. Add glass beads (500 µm) (1:1 / solution:beads by volume).
8. Lysis is performed by repeatedly vortexing the tube for 30 s on maximum setting and bringing them back on ice for 30 s. Continue this process for 5 min.
9. Centrifuge the tube for 10 min at 3000 RPM at 4 °C.
10. Collect the supernatant into a separate 50 mL Falcon tube and centrifuge it for 30 min at 16 000 RCF at 4°C.
11. Decant the resulting supernatant into an eppendorf tube.
12. Resuspend the remaining precipitate in a lysis buffer.

Procedure for Quantitating Sulfhydryl Groups Based on Molar Absorptivity

A. Material Preparation

- Reaction Buffer: 0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA
- Ellman's Reagent Solution: Dissolve 4 mg Ellman's Reagent in 1 mL of Reaction Buffer

B. Measure Absorbance

1. For each unknown sample to be tested, prepare a tube containing 50 µL of Ellman's Reagent Solution and 2.5 mL of Reaction Buffer.
2. Add 250 µL of each unknown to the separate test tubes prepared in step 1. As a blank, add 250 µL of Reaction Buffer to a separate test tube prepared in Step 1.
    - Note: For the unknown(s), make dilutions so that the 250 µL sample applied to the assay reaction has a sulfhydryl concentration less than 1 mM. Concentrations exceeding 1 mM free sulfhydryl will result in high absorbance values and less accurate estimation of the concentration based on the extinction coefficient.
3. Mix and incubate at room temperature for 15 minutes.
4. With a spectrophotometer set to 412 nm, zero the instrument on the blank and then measure absorbance of each sample.
5. Calculate the amount and concentration of sulfhydryls in the sample from the molar extinction coefficient of TNB (14,150 M^{-1} cm^{-1}), as exemplified in Section C.

C. Example Calculation of the Free Sulfhydryl Concentration

A 250 µL aliquot of the unknown mixed with 2.5 mL of Reaction Buffer and 50 µL of Ellman's Reagent Solution gave an absorbance of 0.879 (after subtracting the blank) using a 1 cm spectrophotometric cuvette. Calculate the sulfhydryl concentration in µmoles per mL of unknown. The reported molar absorptivity (molar extinction coefficient, which is

expressed in units of M^{-1} cm^{-1}) of TNB in this buffer system at 412 nm is 14,150.2 Molar absorptivity, E, is defined as follows:

$E = \frac{A}{bc}$ where A = absorbance, b = path length in centimeters, c = concentration in moles/liter (=M)

Solving for concentration gives the following formula: $c = \frac{A}{bE}$

In the present example, A = 0.879, b = 1 cm and E = 14,150 M^{-1} cm^{-1}. Therefore, $c = \frac{0,879}{1(14,150)}$ = 6.21 x 10^{-5} M

This value represents the concentration of the solution in the spectrophotometric cuvette. To calculate the concentration of the unknown sample, it is necessary to account for dilution factors as follows:

The total volume of the solution being measured is

2.50 mL of Reaction Buffer
\+ 0.25 mL of Unknown Sample
\+ 0.05 mL of Ellman's Reagent Solution
2.80mL of solution

These 1.74 x 10^{-7} moles of sulfhydryl in the assay solution were contributed by the original 0.25 mL sample. Therefore, the concentration of free sulfhydryl in the original unknown sample is

$\frac{1.74 \times 10^{-7} \text{ moles}}{0.25 \text{ mL}} \times \frac{1000 \text{ mL}}{\text{L}}$ = 6.96 x 10^{-4} M

Use of SYBR Green dye for measuring helicase activity

Each helicase reaction contained 20 µL 5x helicase buffer (200 mM Tris pH-7.6, 25 mM $MgCl_2$, 20 mM DTT, 125 mM KCl, 10% glycerol and 0.5 µg/µL BSA), 1 mM ATP, 10 µM DNA oligo, 1xSYBR GreenI and the indicated concentration of helicase. Helicase activity was measured by using a plate reader at 492/535 nm, where reactions were incubated in 65 °C for 1 hour. The enzyme activity was then calculated as follows:

$\frac{y-x}{y}$*100, where x and y represent the fluorescence with and without the added helicase, respectively.

ssDNA concentration measurement with Qubit assay kit

1. Prepare the Qubit working solution by diluting the Qubit ssDNA Reagent 1:200 in Qubit ssDNA buffer.
2. Add a working solution to individual assay tubes so that the final volume in each tube after adding a sample is 200 µL.
3. Add each sample to the assay tubes containing the correct volume of Qubit working solution, then mix by vortexing 2-3 seconds. The final volume in each tube should be 200 µL.
4. Allow all tubes to incubate at room temperature for 2 minutes.
5. Measure ssDNA concentration by using Qubit Fluorometer.

LAB SAFETY

Prologue

As it should, safety has always been the number one priority to the Vilnius-Lithuania iGEM team. This year we *swimmingly* extended a great track record set by our predecessors and placed the safety of ourselves and others at the forefront of our projects' aims. The assistance of accomplished scientists and staff at the Life Sciences Center of Vilnius University (VU LSC) allowed us to fulfill the fundamental task of protecting our environment to the best of our abilities. Careful experiment planning and scrutinous work ethics in the laboratory left no chances for any harm to come to our already delicate nature and the ones relying on its prosperity. The planned experiments were presented in detail to our PI and dean's office and they confirmed that our experimental procedure does not have any biosafety concerns in the laboratory.

Lab safety

All laboratories in our country have to be accredited by the Office under the Ministry of Environment in accordance with the requirements of standard LST EN ISO / IEC 17025: 2018. This year our project employed the use of laboratories classified as biosafety level (BSL) 1 and 2. To minimise the risk of cross-contamination, the experiments carried out in BSL-2 laboratory were performed by a single person following strict safety precautions. In addition, the person was supervised by an experienced staff member at all times. All necessary safety and decontamination protocols were obeyed by said person before transitioning between the laboratories.

As per official protocol, all of the laboratory team members underwent detailed safety briefings and training provided by the qualified personnel of VU LSC before any laboratory work had begun. The training included, but was not limited to:

- Proper use of laboratory equipment and devices.
- Briefing on the necessary and proper use of personal protective equipment (PPE).
- Taking the necessary steps towards preventing any fire hazards, including undertaking the official instructions in the event of a fire.
- Following strict hygiene norms (e.g. no food or drinks in the laboratory, long hair must be tied back when doing work of any kind, ensuring proper ventilation of the laboratory spaces, etc.).
- Disposing of biohazardous materials under the controlled supervision of highly trained staff members.
- Steps to be taken and persons to be contacted in the event of spillage of and(or) human exposure to the biohazardous materials.
- Appropriate handling and storage of various hazardous and(or) flammable materials.

In addition, safe work practices were overseen by our PI - Prof. Rolandas Meškys.

Laboratory team members procured or were provided with all the necessary and approved PPE to ensure their safety, including laboratory coats, gloves, eyewear, closed-toe footwear, long-legged trousers, etc. The use of headphones or other hearing-hindering devices were also prohibited. Before entering or leaving the laboratory premises, all necessary sterilisation steps were taken in order to prevent the spread of microorganisms into the environment. Biological safety cabinets were disinfected after each use and deep cleaning was performed at the end of each week.

To prevent cross-contamination of laboratory surfaces and biological safety cabinets proper use of various disinfecting sprays and aseptic technique was employed.

PROJECT DESIGN

General microorganism information

The nature of our project demanded involvement of a single species belonging to the risk group 2 - *Klebsiella pneumonia* (strain KV-3), however we managed to reduce any possible risk of human exposure to a minimum. In fact, the bacteria were only used at the absolutely-necessary stages of project development for proof-of-concept purposes and the final product would not contain or promote the proliferation of any harmful bacteria nor be adverse to the environment. Our PI provided the *Klebsiella pneumonia* along with all the necessary instructions for a safe work approach. As mentioned before, only a single person was working with *K. pneumoniae* in a BSL-2 rated laboratory while also being supervised. The person had no known immunodeficiency and no chronic diseases, which could possibly make him susceptible to the infection. On the days of the experiments the person did not come in contact with any of the other team members.

All other microorganisms used are classified as belonging to risk group 1 and were handled in a BSL-1 rated laboratory. These included well-characterized and non-pathogenic *Escherichia coli* strains (*E. coli* BL21 (DE3), Rosetta (DE3), Arctic Express (DE3), DH5α, DH10B, and ER2566), already present in our lab inventory and used as a chassis for most of our parts.

The remaining part was expressed in a *Saccharomyces cerevisiae* (strains 214, 214Δpep, Gcn2, 214mn 1D and Fh4c). The strains were kindly donated to us by VU BTI EGIS laboratory and transported in an appropriate hermetically sealed packaging, thus no risk of release to the environment was posed. Additionally, when working with the yeast strain a separate biological safety cabinet was used to eliminate any possibility of cross contamination with the other microorganism species.

The last three risk group 1 organisms we used belonged to the family of *Flavobacteriaceae*: *Flavobacterium branchiophilum* (strain 24789), *Flavobacterium psychrophilum* (strain 18644), *Flavobacterium columnare* (strain 3660). All of these microorganisms were obtained from The Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH. These organisms are known to infect animals, however every precaution was taken so as to not release these organisms into the wild.

Specific project design

In its entirety, the project was divided into three main subprojects: detection, treatment and prevention.

Detection

This part of the project was nominated with the task of developing a pathogenic bacteria detection kit based on isothermal helicase-dependent amplification (HDA) and lateral flow assay (LFA) methods. The kit contains no cells and poses no threat of bacterial contamination. The HDA enzymes are also harmless. As long as it would be handled using the instructions provided and disposed of appropriately it presents no threat to the environment.[1]

Treatment

Currently, this is merely a laboratory-contained exercise intended for a scientific advancement of the future treatment capabilities and other applications of GMOs. Regardless, no proteins or other materials that could be released by our designed transfer bacteria are known to cause any harm to the environment nor were they exposed to it. At no point was it considered to apply the use of this treatment method *in natura*. Every precaution was made to prevent the release of GMOs into the environment as per the rules of iGEM and regulations of VU LSC. In an extremely unlikely case of environmental release, the GMOs were equipped with three separate kill-switch mechanisms in order to prevent their spread.

Prevention

The idea of preventing fish disease was to develop an environmentally friendly edible vaccine. Thus, by design, none of the materials it contains are harmful to the aquatic environment or the environment in general. The vaccine contains no cells and only their protein products, carefully purified using protein chromatography and dialysis techniques, which are known to prevent bacterial contamination.[2]

No parts used in the project's development are known to be toxic to humans, animals

[1] Wingfield, P. T. Overview of the purification of recombinant proteins. Current protocols in protein science 80, 6.1.1-6.1.35 (2015).

[2] Nanotechnologies - Part 2: Guide to safe handling and disposal of manufactured nanomaterials (2013).

or plants, thus no risk was placed on the persons working with the cellular products and only the target bacteria would be affected.

Use of harmful reagents and procedures

Some experimental techniques required the use of UV light or usage of compounds, requiring additional safety protocols. All necessary precautions were taken to prevent harm.

UV light: UV light was used to sterilize biological safety cabinets and the equipment contained in them, as well as for DNA imaging in agarose gels needs. Our team members made certain to wear necessary PPE. Due to very few personal close-up encounters with UV light and having taken safety precautions, we completely minimized the possibility of experiencing UV damage and the health risks concerning it. Also, to minimize the contact time with the UV light, we introduced blue light transilluminators to common laboratory procedures.

Ethidium Bromide: This compound is a potent mutagen and is moderately toxic after acute exposure. Therefore, the lab team took it upon themselves to familiarize themselves with the appropriate procedures that are required to handle cases of spills or acute exposures. These procedures included working in a functioning fume hood and wearing personal protective equipment (PPE) to minimize the chance of exposure, as well as extensively studying the material safety data sheet (MSDS) for Ethidium Bromide. We disposed of the product in accord with the disposal protocols and the state safety regulations.

Antibiotics: We used a few main antibiotics for the selection procedure. According to the material safety data sheet (MSDS) for ampicillin, chloramphenicol, and kanamycin, these compounds are known to have hazardous effects at high concentrations and both acute and chronic exposure to humans via inhalation, ingestion, or contact with eyes. Therefore, the team wore personal protective equipment (PPE) when handling the compound, studied the safety protocols, and were equipped with the necessary information to handle spills, acute exposures, and disposed of the product in accord with the disposal protocols as well as the state safety regulations.

Acrylamide: In a laboratory setting, acrylamide is a widely used compound, necessary for many research procedures. Unfortunately in its pure form it is highly toxic and mutagenic. As with any other reagent, however, our team took all necessary steps in ensuring its safe use by strictly adhering to the manufacturer's safety data sheet along with appropriate disposal of the gel.

Once again it should be reiterated that all of the organisms, their products and other reagents were handled conscientiously and by abiding all the pertinent safety guidelines

provided by their relevant providers. At no point did our team members deviate from the strict safety standards and policies presented to us by the VU LSC, iGEM and the suppliers.