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🌐 Two-Stage Dynamic Control Protocol for Cloning, Autoinduction, Autolysis, and Purification of Nanobodies from the E. coli Cytoplasm

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We use this protocol and it's working

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Disclaimer

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Abstract

This protocol aims to offer a straightforward and efficient procedure for cloning nanobodies (VHHs) into a vector designed for phosphate dependent auto inducible expression in the E. coli cytoplasm. We provide step-by-step instructions for cloning, autoinduction, simple cell lysis, and the purification of VHHs through filtration. This protocol is designed to be accessible, less empirical, and adaptable to various parameters such as expression time and temperature. It represents a robust and versatile approach for high titer VHH expression and purification in E. coli, making it suitable for a wide range of applications in nanobody research.

Two-Stage Dynamic Control Protocol for Cloning, Autoinduction, Autolysis, and Purification of Nanobodies from the E. coli Cytoplasm

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Reagents

■ Plasmids

- Addgene plasmid pHCKan-yibDp-GFPuv (Plasmid #127078)
- Addgene plasmid pHCKan-Empty vector (Plasmid #202466)
- Addgene plasmid pCOLA-Spec-yibDp-Erv1p (Plasmid #212094)
- Addgene plasmid pCOLA-Spec-EV (Plasmid #212095)

■ DNA/g-block(s)

- Codon optimized VHH g-block(s)

■ Oligos

- JNH35: TAATGAGGATCCCCGGCTTATCG
- JNH36: CATAGATTATCCTCCTACACAGAAGTTATCCTGACGTTTAA
- SL1: CAGTCCAGTTACGCTGGAGTC
- SR2: GGTCAGGTATGATTTAAATGGTCAGT

■ Molecular biology reagents

- Nuclease free water
- Q5 polymerase (NEB, Cat# M0492S)
- Taq polymerase (Roke Bio, Cat# RE-005TMMD400)
- DpnI (NEB, Cat# R0176S)
- NEBuilder[®] HiFi DNA Assembly Master Mix (NEB, Cat# E2621S)
- Glycerol (molecular biology grade)

■ E. coli strains

- E. cloni (or any cloning strain)
- JNH_Redox6AL (DLF_S0025, trxB-das4:tetR, Δgor-phoA-dsb:purR, Autolysis:apraR) (Roke Bio, Cat# RSA-003)

■ Media components

- Yeast extract (Thermo Fisher, Cat# 288620)
- Ammonium sulfate
- Citric acid
- Fe (II) Sulfate
- MgSO₄
- CaSO₄
- Glucose
- MOPS
- Thiamine-HCl
- Casamino Acids
- Agar A
- Tryptone

- NaCl
- H₂SO₄
- CoSO₄·7H₂O
- CuSO₄·5H₂O
- ZnSO₄·7H₂O
- Na₂MoO₄·2H₂O
- H₃BO₃
- MnSO₄·H₂O
- **Autolysis buffer components**
- Triton-X100 (molecular biology grade)
- Tris base
- MgCl₂
- **Materials**
- PCR tubes
- 1.7mL microcentrifuge tubes
- Electroporation cuvettes (1mm)
- 250 mL vented baffled flasks (VWR, cat. no. 89095-270)
- 30kDa MWCO Amicon Ultra-0.5 Centrifugal Filter (Millipore Sigma, SKU UFC503024)

Equipment

- Pipettes
- Thermocycler
- Autoclave
- Centrifuge
- Electroporator
- Plate incubator
- Orbital shaking incubator
- -80°C freezer

Procedure

Competent Cell Preparation Note:

- Prepare competent cells for both E. coli strains (E. cloni and autoDC Redox) before starting the protocol. While the protocol outlines transformations with electrocompetent cells, it can be adapted for use with chemically competent cells.

Sequence design and Codon Optimization

1. Design a g-block to express your VHH of interest. Ensure that the sequence is codon-optimized for expression in E. coli.
- The start codon is in bold below and stop codons are in bold and underlined.
 - Recommended homology arms: TTCTGTGTAGGAGGATAATCT**ATG** - [codon-optimized VHH sequence] - **TAATGA**GGATCCCCGGC.

VHH Cloning and Sequencing

Preparing Template Plasmid (Day 1- Day 2)

1. Inoculate a 5mL starter culture with pHCKan-yibDp-GFPuv from Addgene (Plasmid #127078), supplemented with 35µg/mL of Kanamycin sulfate. Incubate 16 hours (overnight) at 37°C.
2. Use a standard miniprep to isolate pHCKan-yibDp-GFPuv for use as a PCR template.

PCR Amplification and Dilutions (Day 2)

1. Perform 3 (1:10) serial dilutions of the pHCKan-yibDp-GFPuv plasmid.
2. Set up 3 PCR reactions, one for each serial dilution, using 1 µL of template plasmid per reaction.

PCR Amplification Parameters (Day 2)

1. Amplify pHCKan-yibDp-GFPuv with primers JNH35 and JNH36 using NEB Q5 polymerase with the following parameters:
 - JNH35: TAATGAGGATCCCCGGCTTATCG
 - JNH36: CATAGATTATCCTCCTACACAGAAGTTATCCTGACGTTTTA
 - Annealing temperature: 69°C
 - Extension time: 60 seconds

Verification and DpnI Digestion (Day 2)

1. Use gel electrophoresis to verify that your PCR product size
 - **Anticipated results:** ~2.1kb fragment.
1. DpnI digest the PCR reaction that yielded the product with the lowest template concentration. Add 1µL of DpnI directly to the PCR tube and incubate at 37°C for 1 hour.

Gibson Assembly (Day 2)

1. Perform the Gibson assembly according to the NEBuilder® HiFi DNA Assembly Master Mix protocol.

Setting Up DpnI Negative Control (Day 2)

1. Set up a negative control by diluting your DpnI-digested PCR reaction in water to normalize to the same volume as your Gibson assembly.
2. Perform a 1:3 dilution of the Gibson assembly and the DpnI negative control with nuclease-free water.

Electroporation and Cell Recovery (Day 2)

1. On ice, aliquot 50µL of electrocompetent E. cloni (Lucigen, or any cloning strain) into sterile 1.7mL microcentrifuge tubes. Set up 1 tube per reaction.
2. Add 1µL of each dilution (VHH Gibson assembly or DpnI digest negative control) to 50µL of electrocompetent E. cloni.
3. Add the contents of each mixture to a separate 1 mm electroporation cuvette, pre-chilled on ice.
4. Electroporate each cuvette and immediately use 950µL of SOC to transfer the cells from the electroporation cuvette to a sterile 1.7mL microcentrifuge tube.
5. Recover the cells for 1 hour in a 37°C orbital shaker.
6. Plate each transformation on low-salt LB Kanamycin plates, and perform multiple dilutions to isolate single colonies. Plate the same volume of the Gibson assembly and DpnI negative control. Incubate plates overnight at 37°C.

Colony PCR and Confirmation (Day 3)

1. Compare the DpnI negative control plate to the VHH Gibson assembly plates.
 - **Anticipated results:** Negligible colonies on the DpnI plate.
1. Perform colony PCR with Taq polymerase, according to the manufacturer's protocol, to confirm the size of your insert. Use pHCKan-yibDp-GFPuv as a process control.
 - Use the following parameters for colony PCR:
 - SL1 primer: CAGTCCAGTTACGCTGGAGTC
 - SR2 primer: GGTCAGGTATGATTTAAATGGTCAGT
 - Annealing temperature: 52°C
 - Extension time: 60 seconds/kb.
1. Use gel electrophoresis to confirm colonies with the correct insert size.
 - **Anticipated results:** pHCKan-yibDp-GFPuv process control fragment is ~1.1kb

Plasmid Isolation and Sequencing (Day 3 - Day 4)

1. After colony PCR confirmation, use a single colony to inoculate a 5mL starter culture, supplemented with 35µg/mL of Kanamycin sulfate. Incubate overnight at 37°C.
2. Use some of the starter culture to make a glycerol stock of each strain for future use. Mix the culture with sterile 50% glycerol in a 1:1 ratio. Store the glycerol stock at -80°C for long-term preservation.
3. Use a standard miniprep to obtain the plasmid from the remaining culture.
4. Use SL1 and SR2 primers to sequence the insert.

Transformation into Production Strain

Starter Cultures and Plasmid Isolation (Day 4 - Day 5)

1. Ensure that you have an isolated stock for each plasmid listed below.
 - Addgene plasmid pCOLA-Spec-yibDp-Erv1p (Plasmid #212094)
 - Addgene plasmid pCOLA-Spec-EV (Plasmid #212095)
 - Addgene plasmid pHCKan-Empty vector (Plasmid #202466)
 - Addgene plasmid pHCKan-yibDp-GFPuv (Plasmid #127078)
 - pHCKan-VHH - cloned in the previous section
1. Make a 5mL starter culture for each plasmid from the list that you still need.
 - For pHCKan plasmids, supplement the culture with 35µg/mL of Kanamycin sulfate.
 - For pCOLA plasmids, supplement the culture with 50 µg/mL of Spectinomycin.
1. Incubate the starter cultures overnight in a 37°C shaker.
2. Utilize a standard miniprep to isolate each plasmid.

Preparing AutoDC Redox Transformations (Day 5)

1. On ice, aliquot 50µL of electrocompetent autoDC Redox into sterile 1.7mL microcentrifuge tubes.
2. Prepare a microcentrifuge tube for each condition outlined in the table below. Add 1µL of the pHCKan and the pCOLA-Spec plasmid to the respective tube.

	pHCKan	pCOLA-Spec
		Purpose



	pHCKan-yibDp-GFPuv	pCOLA-Spec-EV	Positive control
	pHCKan-Empty vector	pCOLA-Spec-EV	Negative control
	pHCKan-Empty vector	pCOLA-Spec-Erv1p	Negative control
	pHCKan-VHH	pCOLA-Spec-Erv1p	VHH expression

1. Add the contents of each mixture to a separate pre-chilled electroporation cuvette

Electroporation and Cell Recovery (Day 5)

1. Electroporate each cuvette and immediately use 950µL of SOC media to transfer the cells from the electroporation cuvette to a sterile 1.7mL microcentrifuge tube.
2. Recover the cells for 1 hour in a 37°C shaker.
3. Plate each transformation on low salt LB plates supplemented with 35µg/mL of Kanamycin and 50 µg/mL of Spectinomycin. Perform multiple dilutions to isolate single colonies. Incubate overnight at 37°C.

Inoculate Starters (Day 6)

1. Inoculate a 5mL low-salt LB starter for each condition using a single colony, supplemented with Kanamycin and Spectinomycin.

Glycerol Stock Preparation (Day 7)

1. Prepare a glycerol stock for each strain, ensuring their availability for future use as detailed in step 22.
 - **Note:** the remaining culture will be used to inoculate flasks in the subsequent section

VHH expression with autoinduction

Preparing AB Autoinduction Media (Day 6)

1. Make AB autoinduction media([Menacho-Melgar, Ye, et al. 2020](#)). Media stock solutions are prepared with ultrapure water as follows:
 - 3 M Ammonium sulfate solution. Autoclave and store at RT.
 - 100 g/L citric acid. Autoclave and store at RT.
 - 1 M Potassium 3-(N-morpholino) propanesulfonic Acid (MOPS), adjust to pH 7.4 with KOH. Filter sterilize (0.2 µm) and store at RT.
 - 2 M MgSO₄ and 10 mM CaSO₄ solutions. Filter sterilize (0.2 µm) and store at RT.
 - 50 g/L solution of thiamine-HCl. Filter sterilize (0.2 µm) and store at 4°C.
 - 500 g/L solution of glucose, dissolving by stirring with mild heat. Cool, filter sterilize (0.2 µm), and store at RT.
 - 100 g/L yeast extract, autoclave, and store at RT.
 - **Note:** use yeast extract from Thermo Fisher, Cat# 288620
 - 100 g/L casamino acid, autoclave, and store at RT.
 - 500X Trace Metal Stock Solution TM1: Prepare a solution of micronutrients in 1000 mL of water containing 10 mL of concentrated H₂SO₄, 0.6 g CoSO₄·7H₂O, 0.5 g CuSO₄·5H₂O, 0.6 g ZnSO₄·7H₂O, 0.2 g Na₂MoO₄·2H₂O, 0.1 g H₃BO₃, and 0.3 g MnSO₄·H₂O. Filter sterilize (0.2 µm) and store at RT in the dark.

- Prepare a fresh solution of 40 mM ferric sulfate heptahydrate in water, filter sterilize (0.2 μ m) before preparing media each time.
- 1. Prepare the final working medium by aseptically mixing stock solutions based on the following table in the order written to minimize precipitation. Adjust the media pH to 6.8 and filter sterilize (0.2 μ m) into a pre-sterilized bottle.
- **Note:** Record the date that the AB media is prepared. AB media is stable for at least 30 days at room temperature. Additional stability tests are needed to verify the shelf life past 30 days.

Ingredient	Stock Concentration	Volume in 1 L (mL)	Final Concentration
Ammoniumsulfate	3 M	13.6	40.8 mM
Citric acid	100 g/L	2.5	0.25 g/L
Trace Metals TM1	500 X	5.6	2.8 X
Fe (II) Sulfate	40 mM	2.4	0.096 mM
MgSO ₄	2 M	4.35	8.7 mM
CaSO ₄	10 mM	7.08	0.0708 mM
Glucose	500 g/L	90.0	45 .0 g/L
MOPS	1 M	200.0	200 mM
Thiamine-HCl	50 g/L	0.2	0.01 g/L
Yeast Extract	100 g/L	62	6.2 g/L
Casamino Acids	100 g/L	35	3.5 g/L

Shake Flask Preparation and Incubation (Day 7)

1. For each condition, inoculate 20 mL of AB autoinduction media with 200 μ L of the overnight LB culture. Supplement the media with Kanamycin and Spectinomycin in pre-sterilized vented baffled 250mL Erlenmeyer flasks (VWR, cat. no. 89095-270).
2. Incubate the flasks at 37°C with agitation at 150 r.p.m., using a 50 mm diameter shaker, for 24 hours.

Shake Flask Harvest (Day 8)

1. After 24 hours, measure the OD 600nm. Save an aliquot (~100 μ L) to analyze the whole cell protein profile.
 - **Anticipated result:** OD 600nm > 16
 - **Note:** Flasks, incubated at 30°C and agitated at 150 rpm with a 50 mm diameter shaker, should be incubated for 48 hours after inoculation (Day 9). Ensure that the OD

600nm reaches > 16.

1. After verifying the OD 600nm > 16, harvest cells by centrifugation at 4200 r.p.m. for 10 minutes at 4°C.

Cellular autolysis (Day 8)

1. Perform cellular autolysis as previously described ([Menacho-Melgar, Moreb, et al. 2020](#)). Resuspend the cell pellets in autolysis buffer, which consists of 20mM Tris base, pH=8.0, 2mM MgCl₂, and 0.1% Triton-X100, to a volume equal to 1/10th of the original flask volume (2mL). This buffer can be supplemented with protease inhibitors.
2. To lyse the cells, follow these steps:
 - Freeze the resuspended cells at -80°C for at least 2 hours.
 - Thaw the cells at room temperature.
 - Incubate the thawed cells at 37°C for 2 hours.
1. Isolate the soluble fraction using two rounds of centrifugation:
 - Round 1: Centrifuge at 4200 r.p.m. for 10 minutes at 4°C.
 - Round 2: Centrifuge at 16,000 r.p.m. for 20 minutes at 4°C.
1. Measure the protein concentration in the supernatant using a standard Bradford assay and evaluate VHH expression with SDS-PAGE.
 - **Anticipated results: Lysed samples should have a protein concentration >10 mg/mL. In the SDS-PAGE results you should see a band that corresponds with the size of your VHH that is distinctly absent in the negative controls. If the GFP control is included in the SDS-PAGE, GFP appears at ~27kDa.**

Filtration purification (Day 8)

1. Dilute each sample to a concentration of 1 mg/mL of total protein content with autolysis buffer that does not contain Triton X-100 (20mM Tris, pH=8.0, 2mM MgCl₂) before proceeding with the filtration step. This dilution is crucial to prevent filter fouling.
2. Use 30kDa MWCO Amicon Ultra-0.5 Centrifugal Filters (Millipore Sigma, SKU UFC503024) for the filtration process.
3. Pre-soak the filter membrane for at least 10 minutes with autolysis buffer that does not contain Triton X-100 (20mM Tris, pH=8.0, 2mM MgCl₂). Remove the buffer immediately before adding the sample.
4. Add the normalized sample to the filter and proceed to centrifuge each sample in a tabletop centrifuge for 30 minutes at 14,000 rcf and 4°C.
5. Analyze both the permeate and retentate fractions using SDS-PAGE to determine the purity and separation of the protein.

Troubleshooting

- High Background (pHCKan-yibDp-GFPuv) Screening pHCKan-VHH Clones:
- **Issue:** Template concentration going into the PCR is too high.
- **Solution:** Perform additional serial dilutions before adding the template to the PCR.
- **Solution:** Check the integrity of DpnI and, if necessary, order a new supply.
- No Colonies on DpnI Negative Control Plate or the pHCKan-VHH Plate:

- **Issue:** Low efficiency competent cells.
- **Solution:** Check the transformation efficiency of your competent cells with pHCKan-yibDp-GFPuv.
- **Issue: Low concentration of the assembled plasmid.**
- **Solution:** Perform a standard clean & concentrate on your gibson assembly rather than a dilution before transformation.
- No Protein Induction:
- **Issue:** The GFP process control (autoDC Redox with pHCKan-yibDp-GFPuv & pCOLA-Spec-Empty vector) does not induce.
- **Solution:** Follow the time course of protein expression, quantify the OD 600 nm and fluorescence.
- **Solution:** Optimize the fill volume of the flask to maximize GFP expression.
- Low VHH Soluble Expression:
- **Issue:** Band of anticipated VHH size does not appear on the SDS-PAGE gel.
- **Solution:** Check the whole cell protein sample to ensure VHH expression is induced.
- **Solution:** Some VHHs have better soluble expression at lower temperatures. Consider decreasing the temperature and include the GFP process control to determine optimal protein expression under low temperature conditions. Note that decreasing the temperature may require prolonged incubation periods to ensure cells reach the required biomass levels for induction.

Time Taken

Once all materials and reagents are obtained, this protocol spans 8 days to successfully clone, express, and purify VHHs tailored for various in vitro applications. Each phase of the protocol is labeled with the corresponding day, ensuring a streamlined and comprehensive procedure.

Anticipated Results

Anticipated results are provided throughout the protocol at relevant steps to assess the success of each procedure.

Additional Declarations

Michael Lynch, Romel Menacho-Melgar, and Jennifer Hennigan have financial interests in Roke Biotechnologies, Inc.

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