Protocol: Colony PCR

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The PCR protocol was originally provided by AMPLIQON. Last year's team has added additional details, clarifications, and tips for practical use in the lab.

Additional changes have been made, to fit our workflow and using a different PCR Kit (Red Load Taq by Jena Bioscience).

⚠️ **Important:** Please read through the entire protocol before starting. This will help avoid mistakes and ensure reliable results.

Overview of Colony PCR

Day 1

1. Pick colonies and streak onto a master plate for backup. Incubate at 37°C.
2. Prepare 20 µL PCR mix per colony in PCR tubes.
3. Use a sterile inoculation loop/pipette tip to pick a small colony (1–2 mm) and dip directly into the PCR mix (do not overdo it, if the solution is cloudy, it's too much).
4. Run PCR.
5. Run gel electrophoresis to check for amplification. A positive result shows a band at the expected size. If no band is visible, the insert is likely missing (one primer binds the insert, the other the plasmid).
6. From positive results, inoculate LB tubes (5 mL each) using clones from the master plate (at least 3 clones per construct).

Day 2

1. Plasmid miniprep from overnight cultures (see separate protocol).
2. Prepare sequencing samples: mix plasmid DNA + sequencing primers + water, following the sequencing kit instructions.
3. Drop off sequencing samples at BMC. Results typically arrive within one to two days.

Day 3

1. Analyze sequencing results. Match results to clone positions on the master plate.
2. (Optional) Streak fresh plates using clones confirmed to have the correct insert.

Part A. Picking Colonies (Day 1)

Important: Keep Track of Which Colony Is Which!

Before you start, prepare a plate map (a sketch or printed grid representing your master plate) and assign each colony a number or code.

1. Pick small colonies (~1–2 mm diameter) using a sterile pipette tip. Don't pick too large of a colony. Too many bacteria can inhibit your PCR reaction or cause non-specific products to show up on your gel.
    a. Write down which colony was picked and which PCR tube it went into.
    b. Label your PCR tubes clearly to match this record.
2. Streak onto a clearly labeled master plate for backup (LB Agar with Ampicillin) and incubate **agar side up** at 37 C over the day.
    a. A *master plate* is a fresh agar plate (with the same antibiotic as your original selection plate) where you streak each colony you pick. It serves as a backup. After PCR, if you find out a certain colony contains the correct insert, you can go back to the master plate to grow that colony again.
3. Place the same inoculation loop into PCR tubes pre-loaded with PCR reaction mix (20 µL) (see part B).
4. Swirl it around to fully resuspend cells.

Part B. PCR Protocol (Day 1)

- Set up reaction mixtures in an area separate from where DNA preparation or product analysis is performed.
- Work on ice at all times.

1. Thaw Red Load Taq Master (5x) and primer solutions. Thaw completely and mix thoroughly to avoid localized salt concentrations.

⚠️ **Important:** Make sure to be super careful so that you pick the right primers for the colony PCR reaction (see Part C below).

Prepare a reaction mix for a final volume you are going to use. Each PCR tube should contain 20 µL in total, so the volume you prepare depends on how many tubes you are going to do.

Multiply Table 1 (which is adapted for 1 reaction). For pipetting errors, prepare one more than you think you will need. Don't forget the controls (see part D)!

Table 1: Reaction mixture using Taq 2x Master Mix RED

Component	***Vol./reaction***	***Final Concentration***
Red Load Taq Master (5x)	4 µL	1x
Primer Forward (10 µM)	0.8 µL	0.4 µM
Primer Reverse (10 µM)	0.8 µL	0.4 µM
Template DNA	0 µL	0
H_2O	14.4 µL	Fill up to reach final volume 20 µL
Total volume	20 µL	-

3. Mix the master mix thoroughly and dispense 20 µL into each PCR tube, provided you are testing the same genetic construct. Mix gently by pipetting up and down.
4. Add template DNA (i.e., picked colonies) into the tubes containing the master mix by dipping the inoculation loop into the tube (see part A). If the mix looks cloudy, you picked too many bacteria. It should remain mostly clear.
5. Program the thermal cycler as shown in Table 2. Adjust annealing temperature based on primer melting temperature (typically 3–5°C below Tm). See Part C for primer information.

Table 2: Thermal Cycler Program (based on Red Load Taq Protocol)

Step	Duration	Temperature	Cycles
Cell Lysis	5 min	95°C	1
Denaturation	30 s	94°C	30
Annealing	30 s	X°C	
Elongation	X min*	72°C	
Final Elongation	5 min	72°C	1
Hold	∞	4°C	

***** *Elongation This depends on the length of the amplicon you are amplifying. As a rule of thumb the Red Load Taq DNA polymerase can amplify 1 kbp per minute at optimal conditions.*

6. Place tubes in the thermal cycler and start the PCR.
7. After amplification, run gel electrophoresis (see separate protocol). The Red Load Taq Master Mix already contains dye, so you can load the PCR products directly.
8. Analyze gel results!

What should you expect?

- Positive result (correct insert): Band of the expected size.
- Negative result (no insert): No band or an incorrect size.
- This PCR uses one vector-specific primer and one insert-specific primer, so if the insert isn't there, you won't get a product.
- Beware of false positives. Just because you get the expected sized PCR product doesn't mean there aren't mutations in your insert. Make sure to submit multiple positive clones for sequencing to verify the insert sequence.
- When analyzing your gel results, refer back to your plate map. This allows you to identify exactly which colony gave a correct insert, so you can go back to the correct streak on the master plate and grow it up again.
- As an extra control, you could add three primers (the two pET11a primers as well as one of the primers on the gene). If there is no insert, only a small part of the plasmid will be amplified.

9. For each clone with a correct band, pick a colony from the master plate (not the original plate).
10. Inoculate into a 5 mL LB Medium tube with antibiotics.
11. Grow overnight at 37°C with shaking.

Part C. Primers for Colony PCR (Day 1)

Table 3. Annealing temperature and expected size for primers and amplicons

Primers	***Annealing temperature***
T7pro_fw	60
T7ter_rev	57
AKR1D1_CH_forward	60
AKR1D1_CH_reverse	61
ci2350_CH_forward	60
ci2350_CH_reverse	59

Part D. Positive and negative controls (Day 1)

Positive Control

- A good positive control is bacteria transformed with the **same vector backbone** (that you know works).
- If it doesn't amplify, something is wrong with your PCR setup or primers.
- Note: Johan usually doesn't use a positive control, so it's not required unless the Colony PCR hasn't worked multiple times.

Negative Control

- Use sterile water instead of a bacterial colony in one of the PCR tubes.
- This control contains everything except the actual colony you're trying to test!
- This checks for contamination in your reagents or setup.
- You can also use untransformed bacteria of the same strain as a negative control (but water is usually enough).

Part E. Plasmid Prep and Sequencing (Day 2)

Step 1: Plasmid Prep

- Use your standard miniprep protocol to isolate plasmid DNA from each overnight LB culture. Plasmid prep means extracting circular DNA (plasmids) from bacteria so you can analyze or sequence it.

Step 2: Prepare DNA for Sequencing

1. Mix plasmid + sequencing primer(s) + water.
2. Follow the instructions provided by your sequencing kit or sequencing service.
3. Take your samples to BMC. If you post in the morning, results typically come back the next day.

Mix2Seq Kit

IMPORTANT: For Sequencing primers and isolated plasmid DNA, use Reverse plasmid primer (Rev_pET-11a primer).

1. In the sequencing tube provided in the Mix2Seq add 5 µL of;
    a. Isolated plasmid DNA up to 15 kbp at a concentration of 50-100 ng/µL or
    b. PCR products; 150-300 bp at 1 ng/µL, 300-1000 bp at 5 ng/µL, 1000-3000 bp at 10 ng/µL
2. 5 µL of sequencing primers at a concentration of 5µM and top off with dH2O until you reach the minimum volume of 10 µL.
3. Write down the qr-code number provided on your tube and what sample it is. Put the samples in a brown paper (same cupboard as the kit) bag and drop it off at the dropbox situated at the entrance of Ekologihuset.
4. Log into Eurofins Genomics (https://eurofinsgenomics.eu/) and label your samples under the “My Sanger Kits & Barcodes” tab.

4 little steps to optimum results



Your template should consist of **5 µl purified DNA** in either of the following concentrations:
Plasmid DNA up to 15kbp: **50-100 ng/µl**
PCR products: 150-300 bp: **1 ng/µl**; 300-1000 bp: **5 ng/ µl**; 1000 - 3000 bp **10 ng/µl**



Add **5 µl** of your SeqPrimer with a concentration of **5 pmol/µl (5 µM)**.



Ensure that the total volume of your DNA / primer mix is at least **10 µl**.



Pipet your premixed sample in one of the **Mix2Seq tubes** and seal the tube with the **blue or yellow lid** from the enclosed **cap pad**.

Do not forget to **note the barcode number on the side of the tubes** before you send us your samples!

Conditions
DNA templates must be purified. Sequencing reactions cannot be repeated and all reactions will be charged. Samples are not stored. For samples containing difficult-to-read streches (GC-rich, hairpin structurs...) we recommend our TubeSeq Service.

Part F. Analyzing results (Day 3)

1. Check your sequencing results (For the Mix2Seq kit you can find the results at the Eurofins Genomics website).
2. When analyzing your sequencing results, refer back to your plate map. This allows you to identify exactly which colony gave a correct insert, so you can go back to the correct streak on the master plate and grow it up again.
3. If a colony has the correct insert and clean sequencing, streak it onto a new plate for long-term storage or further use.
