

miniPCR Sleep Lab[™] - *Morning lark or night owl*?

Getting started

1.	Synopsis	2
2.	Materials needed	4
3.	Lab setup	6
Stude	ents guide	
4.	Background	9
5.	Laboratory guide	13
6.	Study questions	22
7.	Morning-Eveningness Questionaire	27
8.	Extensions	32
Instru	uctors guide	
9.	Expected results	33
10.	Citations and additional resources	34
11.	Learning goals and skills developed	35
12.	Standards alignment	36
13.	Ordering information	37

14. About miniPCR Learning Labs[™]38

1. Synopsis

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Are you a night owl? Or a morning lark? The answer may be in your genes...

This lab allows students to test their own genotypes at the **circadian clock** gene *Per3* locus that has been associated with sleep behavior preferences in humans¹. The coding region of this gene contains a **VNTR** (variable number tandem repeat) that is polymorphic (variable) across people. In one study, a *Per3* allele carrying 4 copies of this repeat was found at higher frequencies in people with a **preference for evening activity**, while the allele with 5 copies of this repeat was found more frequently in individuals with a **preference for morning activities**².

In this lab students will get to assess their own *Per3* genotypes using PCR and gel electrophoresis, while also assessing their sleep phenotypes by answering a questionnaire about their own *chronotypes* (circadian preferences.)

As is true for many postulated genetic associations, the evidence for a link between *Per3* genotypes and sleep phenotypes is not yet definitive – while some studies have shown association between this VNTR and sleep^{2,3}, others were unable to reproduce it⁴.

Your work is needed! We hope that collective data gathered in your classroom can help shed light on this possible association between *Per3* genotypes and circadian phenotypes. You'll participate in an authentic open inquiry investigation on the genetic underpinnings of sleep.

- Techniques utilized: micropipetting, DNA extraction, PCR, gel electrophoresis.
- **Time required:** one 120-minute block or two 45 minute periods.
- **Suggested skill level:** familiarity with DNA amplification concepts, basic familiarity with micropipetting techniques, statistical analysis.



Help your students build proficiency in pipetting, PCR, and gel electrophoresis with additional instructional videos, worksheets, and activities available at: https://www.minipcr.com/tutorials/.

For answers to the lab study questions and extensions, email answers@minipcr.com. Please include the name of the lab, as well as your name, school, and title in the body of the email.



Teacher's Note:

In establishing a genetic association, it is important to remember that an individual student's phenotype is not strongly predictive of their individual phenotype. Indeed, that is a major take home message of this lab. Even if the *Per3* is associated with morning or evening wakefulness, that association can only be seen when looking at averages of large data sets. A major lesson of this lab is that genotype and phenotype are not always linked as tightly as people would like to believe. That individual students' genotype and phenotype don't "match" as they might expect can serve as a valuable starting point for discussions about complex phenotypes, environmental inputs, and genetic determinism.

2021 primer update: In 2021, the Sleep Lab primers were updated for improved *per3* gene detection. It is essential that you check the lot number on your kit and use the appropriate PCR program. Refer to page 15 for detailed information.

¹ Ebisawa T, *et al.* "Association of structural polymorphisms in the human period3 gene with delayed sleep phase syndrome." EMBO Rep. 2001 Apr;2(4):342-6.

² Archer SN, *et al.* "A length polymorphism in the circadian clock gene Per3 is linked to delayed sleep phase syndrome and extreme diurnal preference." Sleep. 2003 Jun 15;26(4):413-5.

³ Viola AU, *et al.* "PER3 polymorphism predicts sleep structure and waking performance. Curr Biol. 2007 Apr 3;17(7):613-8. Epub 2007 Mar 8.

⁴ Osland TM, *et al.*, Association study of a variable-number tandem repeat polymorphism in the clock gene PERIOD3 and chronotype in Norwegian university students. Chronobiol Int. 2011 Nov;28(9):764-70.

Materials needed

Supplied in Kit (KT-1005-01)

Reagents and supplies	Amount provided in kit	Amount needed per student	Storage	Teacher's checklist
5X EZ PCR Master Mix, Load Ready™	240 µl	5 µl	Freezer	
Sleep Lab Primers	960 µl	20 µl	Freezer	
X-Tract™ DNA Extraction Buffer	2400 µl	50 µl	Freezer or room temp.	
100 bp DNA Ladder, Load Ready™	100 µl	12 μl per gel	Freezer	

Sold Separately in Learning Lab Companion Kit (KT-1510-01)

This lab requires reagents for running and visualizing DNA samples on a 2% agarose gel with a fluorescent DNA stain (e.g., SeeGreen[™] or GelGreen[®]). The Learning Lab Companion Kit provides enough electrophoresis reagents for 8 groups when using the blueGel[™] electrophoresis system. Gels can also be prepared with agarose tabs or agarose powder. Refer to https://www.minipcr.com/agarose-gel/ for detailed instructions.

Reagents and supplies	Amount provided in kit	Amount needed per lab group	Storage	Teacher's checklist
All-in-one agarose tabs	8	One tab per agarose gel (2% agarose gel)	Room temp., protected from light	
TBE electrophoresis buffer1X working solution	Supplied as liquid concentrate or powder Sufficient to prepare 600 ml of 1X working solution	30 ml of 1X solution per blueGel™ system	Room temp.	
PCR tubes (0.2 ml)	100	2 per student		
1.7 ml plastic tubes	50	5 <u>per group</u>		

Materials needed (cont.)

Supplied by teacher

Reagents and supplies	Amount needed per lab group	Teacher's checklist
PCR thermal cycler: e.g. miniPCR [®] machine	1 One reaction <u>per student</u> Can be shared between groups	
Horizontal gel electrophoresis apparatus: e.g., blueGel™ electrophoresis system	1 Can be shared between groups	
Blue light transilluminator *Note: A blue light transilluminator is integrated in the blueGel™ electrophoresis system.	1 Can be shared between groups	
Micropipettes	1	
 2-20 μl adjustable volume 		
Disposable micropipette tips	At least 5 per student	
Flathead toothpicks for cheek swabs	1 toohtpick <u>per student</u>	
Distilled water for making agarose gels and diluting TBE buffer	60 ml per gel	
Flask or beaker to dissolve agarose		
Microwave or hot plate to dissolve agarose		
Other supplies: • Disposable laboratory gloves • Protective eyewear • Permanent marker • Cup to dispose of tips		



3. Lab setup Planning your time

This lab has 6 stages, and is designed to run in two 45-min class periods, or a single 2-hour block



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Quick guide: Preparatory activities

Suggested for 8 Student Groups (4 Students per group), 32 total samples tested

A. DNA extraction

- Provide each group with 4 flat-end toothpicks
- Each group will need 4 PCR tubes (200 µl)
- Aliquot 250 μ l of X-TractTM DNA Extraction Buffer per group (or 50 μ l per student)

B. PCR set up

- Thaw primers and master mix by placing tubes on a rack or water bath at room temperature
- Label and dispense the following tubes per group (for 4 PCR reactions):
 - X-Tract[™] DNA Extraction Buffer 250 µl (per A above)
 - 5X EZ PCR Master Mix
 - Sleep Lab Primers
- Each lab group will additionally need:
 - One 2-20 µl micropipette
 - Micropipette tips and a small beaker or cup to dispose them
 - 4 PCR tubes (thin walled, 200 μl microtubes)
 - Permanent marker (fine-tipped)

C. PCR programming and monitoring

- Ensure lab benches are set up with a miniPCR $^{\rm TM}$ machine and power supply
- Ensure the miniPCR machines that are going to be monitored through the PCR reaction are connected to a computer or compatible smartphone/tablet

D. Gel electrophoresis

- Gels can be poured in advance of the class (described on page 17)
- 5 Lanes needed per group (4 Test Samples + 1 lane for DNA ladder), e.g. 40 lanes for 32 students.
- Pre-poured gels can be stored in the fridge, in a sealed container or in plastic wrap, in the dark
- If doing the gel run on a different day than the PCR run, completed PCR tubes can be stored in the fridge for up to one week until they are used, or in the freezer for longer-term storage



100 µl





F. Data analysis and interpretation

- Have the banding pattern of the 100bp DNA Ladder handy to help interpret the electrophoresis results (see page 21)
- Have a data sheet available for students to collect class data. We have created a Google Sheet document the that we recommend using with your class for compiling data and calculating statistics. It is available for download at the educational resource section of our website: <u>https://www.minipcr.com/educational-resources/#toggle-id-3</u>
- For simplicity, though the morning-wakefulness score identifies five categories, we have condensed the data collection to just three: morning, evening, and intermediate.

4. Background and significance

Have you ever felt jet-lagged after traveling far from home, and found it hard to sleep? Do you usually find yourself feeling tired at the same time of the day every day? Both of these phenomena are controlled by your *circadian clock*. Circadian clocks are internal timekeepers that regulate our body's physiology and behavior on a cycle that repeats each day. Our internal clock dictates what times of day you feel sleepy, energized, or hungry, and controls important bodily functions such as our blood pressure, body temperature, hormone release, and metabolism. In humans, the circadian clock is set to be a little longer than 24 hours. Other animals, plants, fungi, and even unicellular organisms have circadian clocks too, which helps them anticipate the daily transitions between light and darkness.

Any good clock has two important features. First it needs to keep regular time, and second, it needs to be able to be set and reset. Our circadian clock has both these features. Our clocks are set to roughly 24 hours, but they can also be reset by external factors, especially sunlight; this ensures that your internal timekeeper (naturally a little longer than 24 hours) stays synchronized with the natural day (24 hours). It is also why when you travel to a new time zone your internal clock is initially off, causing jet lag, but within a few days resets to the new time zone you are in. We call this ability of the circadian clock to synchronize to the environment *entrainment*.



A fascinating feature about the circadian clock is that it operates at the cellular level; yes, individual cells in your body can keep and tell time! This is possible because the circadian clock is controlled by a genetic feedback loop, where proteins regulate gene expression with 24-hour periodicity. Circadian clock proteins exhibit *negative feedback*, meaning the proteins produced by the clock genes, in turn, turn off the genes that produced them. Production of circadian clock proteins will therefore continue until the proteins reach a certain concentration in the cell, at which time their production will cease. Over time as these circadian clock proteins are degraded at a regular rate, the concentration of clock proteins will go down until it is low enough that gene expression can resume. Here the cycle will continue – producing

proteins until expression is halted, degrading proteins until expression begins again. Amazingly, the daily cycle of expression and degradation of these proteins has been so fine-tuned by evolution that it closely matches a 24-hour Earth day.

Molecular mechanism of the clock: Transcription/translation feedback loops

We live in an environment that is cyclic due to the Earth's rotation around its axis. Many different living systems have evolved *biological clocks* that can predict the Earth's rotation and the 24-hour light-dark cycle. This biological clock controls metabolism, biochemistry, and many functions inside the body including our activity-rest cycles.

Individual cells in our bodies have internal clocks. In humans and other mammals, the master clock is a tiny structure in the hypothalamus called the *suprachiasmatic nucleus* (SCN). Cells in the SCN (and many tissues in the body) can each oscillate with a ~24-hour period. In the 1990s, mutants helped scientists discover

clock genes (such as *Clock, BMAL, Period*, etc.) which are fundamental in generating circadian rhythms.

We now understand the genetics of circadian behavior in remarkable detail. The molecular mechanism of the clock involves transcriptiontranslation negative feedback loops of multiple genes. The transcription factors BMAL1 and CLOCK form



heterodimers, which activate transcription of Cryptochrome (*Cry*) and Period (*Per*) genes by binding to their promoters. CRY and PER proteins gradually accumulate in the cytoplasm. CRY, PER and other proteins form complexes that translocate to the nucleus and shut down BMAL1–CLOCK mediated expression of the *Cry* and *Per* genes. This transcription/translation negative feedback loop repeats itself every 24 hours inside your cells! To learn more about these and other circadian clock genes, read <u>this review article</u> by circadian clocks geneticist Joe Takahashi.

Genetic variation and the clock

One important gene in these feedback loops is the *Period 3* circadian clock gene (*Per3*). Research has found that there is variation in this gene among humans, i.e. the gene is *polymorphic*. Further studies have found that a specific form of variation in this gene, a *variable number tandem repeat* (VNTR) in *Per3* can affect how people's circadian clocks are set. A VNTR is a short sequence that repeats itself several

times in succession within a gene. In the case of *Per3*, there is a 54-base pair sequence that is repeated 4 times in one allele, and 5 times in another variant. Research has found that carrying 4 copies of this repeat may be associated with a preference for evening activity, while having 5 repeats may be associated with a preference for activities in the morning. **It would seem from these studies that your genes can influence whether you prefer to be a morning lark or a night ow!!**

Since polymorphisms (variation) in this repeat change the length of this gene, the difference between 4repeat and 5-repeat *Per3* genes can be seen in gel electrophoresis. Of course, we first need to amplify (make a lot of copies of) this gene, to make it visible on a gel.

About genetic associations

Most phenotypes are complex traits, with multiple genetic and environmental components (*i.e.*, not determined by a single gene). Genetic association studies are used to find candidate genes or genome regions that contribute to a given trait by **testing for a correlation between that trait and genetic variation**. A higher frequency of a given allele (or genotype) in a sample of individuals who express the trait can be interpreted as meaning that the allele increases the probability of having that specific trait. Associations are difficult to establish unequivocally, and require obtaining large datasets to increase the statistical confidence in the possible association.

Traditional genetics techniques tend to look for mutant or nonfunctional versions of genes to try to determine the function of the gene. While this has been a fruitful approach, until only very recently, it was limited to use on model organisms grown in labs. This limits the ability of scientists to study human genetics in this way. Also, traditional genetics techniques typically do not capture real world variation affecting real world phenotypes. The power of association studies lies in taking human phenotypic variation and being able to associate it with actual genetic variation present in populations.

Associations can be difficult to establish, however, because most traits are controlled by many factors. Let's take a trait like height as an example. While you have no doubt learned about phenotypes being controlled by dominant and recessive alleles, complex traits like height are controlled by hundreds of genes. Each one of those genes has different alleles that may influence you being a little taller or a little shorter. For the vast majority of these we have no idea if an allele is dominant, recessive, or displays some other dominance relationship. You may know that for one particular gene you have an allele that has been associated with increased height. But it's only when you add together the effect of all the alleles for all those genes and include outside influences such as diet that your actual height is established.

This is what we are trying to test today. There are reported associations between the *Per3* alleles and morning or evening preference, while other studies found none. Whether or not this is a real association and how strong an association it may be is still an open question. As is true with all association studies, sufficient sample size and statistical tests are needed to establish a true correlation. In this lab, your class can contribute the data you collect to help establish how important the *Per3* gene is to determining morning or evening preference!

Let's get started with inquiry-based science

In this lab, you will use PCR to amplify a segment of the *Per3* gene (from your own DNA) and gel electrophoresis to directly observe whether you carry the **4-repeat or 5-repeat genotype** in this VNTR within a circadian clock gene. 4-repeat alleles will be amplified as a ~350 base pair fragment, while 5-repeat segments will amplify as a ~400 base pair fragment. We will also take a simple self-assessment questionnaire that will help us determine our *chronotype*, or phenotypic circadian preference (whether you're a morning or evening type.)

We hope that **matching chronotypes to genotypes across many students**, and later aggregating the data, will help us shed some light on the question of this reported genetic association between the *Per3* VNTR and circadian preferences. Through this lab everyone can become a circadian biology researcher!

Please remember that just as is true for many other traits, your circadian clock and sleep patterns are not solely determined by genetics. Modern society is filled with artificial light, caffeine, changing feeding schedules, work or school obligations, and other external signals which interact people's intrinsic sleep behaviors (chronotypes). While your genes may predispose you to certain sleep behaviors, your environment still changes those patterns in ways you may not expect.

Laboratory guide

Protective gloves and eyewear should be worn for the entirety of this experiment.

DNA extraction

For best results, don't eat or chew gum for ~20 minutes prior to cheek cell collection

- 1. You should receive a tube with 50 μl extraction buffer in it. Label the tube with your initials
 - Label tubes on the upper sidewall, as writing on the cap or lower sidewall may rub off.

2. Collect cheek cells

- Gently scrape the inside of your cheek 3-4 times with a flat-end toothpick.
- It shouldn't hurt.

3. Transfer cheek cells to tube with buffer

- Dip the end of the toothpick with your cheek cells in the X-Tract[™] buffer.
- Swirl the toothpick to dislodge the cells.
- Dispose of the toothpick.

4. Cap your tube

5. Incubate your tube for 10 minutes at 95°C

- You can use a miniPCR[®] thermocycler in Heat Block mode, a water bath, or other heat block.
- Note: If using a heat block or water bath, weigh down the lids of your tubes so they don't pop open.

6. Remove your tube from heat and immediately use DNA extract for PCR

• Note: The DNA extract is stable for up to two hours, but the sooner you use it for PCR the better.







PCR setup

- 1. Label a PCR tube (200 μl tube) with your initials followed by "P" for PCR
 - Note: label tubes on the upper sidewall, as writing on the cap or lower sidewall may rub off during PCR.

2. Add PCR reagents to your labeled PCR tube

• Change pipette tips between samples to prevent contamination.

Reagent	Volume
Sleep Lab Primers	20 µl
5X EZ PCR Master Mix™	5 μl
Student DNA sample	3 μl
TOTAL VOLUME	28 µl

Note: EZ PCR Master Mix[™] contains:

• Taq DNA polymerase • dNTPs

- PCR buffer with $\mathsf{Mg}^{\scriptscriptstyle 2*}$ - Gel loading dye

3. Cap the tubes and ensure the reagents mix well

- You may flick each tube with your fingers to ensure proper mixing.
- Gently tap tubes on your bench to collect liquid at the bottom.

4. Place the tubes inside the miniPCR[®] machine

- Press firmly on the tube caps to ensure a tight fit.
- Close the PCR machine lid and tighten it gently.





at each step!

Flick to mix



Tap to collect liquid at bottom





PCR programming

These instructions are illustrated using miniPCR[®] software on a Windows PC. Software interfaces vary slightly by operating system. See the miniPCR[®] User's Guide for more details.

If using a different thermal cycler, PCR protocol parameters should remain the same (step 7).

IMPORTANT NOTE: CHECK YOUR KIT'S LOT NUMBER: Before proceeding further, please check that your Sleep Lab lot number (as labeled on the outside bag) is 211001 or starts with $22____$. In October 2021, Sleep Lab Primers were updated for improved *per3* gene detection. It is essential that you check the lot number on your kit! The protocol below is for kits with lot number 211001 or a lot number that starts with a number ≥ 22 (e.g. $22____$). If your kit has a prior lot number, the annealing temperature should be 65°C instead of 57°C. All other program parameters can remain the same.

- 1. Open the miniPCR $^{\scriptscriptstyle (\!R\!)}$ app and remain on the "Library" window
- 2. Connect your miniPCR[®] thermal cycler to your device using the supplied USB cable or via Bluetooth[®]
 - Note: Bluetooth[®] is only available on certain models. To connect via Bluetooth[®], select the m icon, located by "Devices" at the left of the desktop app or at the top of the mobile app.
- 3. Make sure your miniPCR[®] thermal cycler is plugged in and that the power switch is turned on
 - Note: If your machine begins running a previously loaded protocol, you may stop it by clicking or tapping the "X" symbol in the top left box of the "Monitor" window.
- **4.** While in the "Library" window, click the \oplus button to create a new protocol
 - Button is located in the upper right hand corner of the window.
- 5. Select "PCR" from the drop-down menu
- 6. Enter a name for the protocol; for example: "Sleep Lab"

7. Enter the PCR protocol parameters:

- Initial denaturation 94°C, 30 sec
- Denaturation 94°C, 20 sec
- Annealing 57°C, 20 sec
- Extension 72°C, 20 sec
- Number of cycles 30
- Final extension 72°C, 30 sec

Note: The "Heated lid" slider should be in the on position.





8. Click "Save and run" to start the protocol

- If connected to more than one machine, choose the serial number of the miniPCR[®] thermal cycler you are using. If asked "Do you want to stop the current protocol...?", click "Yes".
- The lights on the front of the miniPCR[®] thermal cycler will blink 3 times to indicate that the protocol has been loaded.
- Note: If needed, you may unplug the USB cable or disconnect Bluetooth[®] once the protocol has been loaded. Even if disconnected from your device, the protocol will continue to completion as normal.

9. Choose "Monitor" window

- The "Monitor" window can be selected on the left column in the desktop app and at the top in mobile app.
- If more than one miniPCR[®] thermal cycler is connected to the same device, choose which machine you would like to monitor using the tabs at the top of the window (desktop app) or bottom of the Library (mobile app).



The miniPCR[®] software allows each lab group to monitor the reaction parameters in real time.

10. When the PCR run has completed (approximately **60** min), app status will show "Finished" and the red, yellow, and green LEDs on your miniPCR[®] thermal cycler will light up and stay on

Be careful not to touch the metal lid which may still be hot.

- 11. PCR product is stable at room temperature for several days. For longer term storage, move tubes to a fridge or freezer
 - Tubes may remain inside the miniPCR[®] thermal cycler for several days following protocol completion.

Laboratory guide



Protective gloves and eyewear should be worn for the entirety of this experiment.

Gel electrophoresis - Pouring gels (before or during class period)



Gels can be prepared up to three days ahead of time and stored at ambient temperature, covered in air-tight plastic wrap and protected from light.

You will need one lane per student plus one lane for ladder per gel.

These instructions are designed for use with the blueGel[™] electrophoresis system by miniPCR bio[™]. If using another electrophoresis system, these instructions may need to be adjusted according to the manufacturer's instructions.

1. Prepare 1X TBE buffer (to be completed by teacher in advance)

- TBE buffer is often provided as liquid concentrate or powder.
- Follow manufacturer's instructions to prepare 1X TBE buffer solution.
- **2.** Prepare a clean and dry casting platform with a gel tray and comb
 - Place the clear gel tray in the white casting platform.
 - Place a well-forming comb at the top of the gel tray.
- 3. Prepare a 2% agarose solution with a fluorescent DNA stain (e.g., SeeGreen[™] or GelGreen[®]) using the method indicated by your instructor



IMPORTANT NOTE: There are several ways to prepare agarose gels

- Scan the QR code for detailed instructions on how to prepare agarose gels.
- Both written and video instructions are available.



www.minipcr.com/agarose-gel/



- 4. Pour the agarose solution into the prepared casting platform with a gel tray and comb
 - The agarose solution should cover the bottom of the gel tray and the bottom 3 mm of the comb (roughly the bottom 1/3 of the comb).
- **5.** Allow gel to solidify completely and remove the comb by pulling firmly upwards
 - Gels will typically be ready in about 10 minutes.
 - Gel is ready when cool and firm to the touch.



Student's Guide

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Student's	Guide	
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Protective gloves and eyewear should be worn for the entirety of this experiment.

Gel electrophoresis - Running the gel

These instructions are designed for use with blueGel[™] electrophoresis system by miniPCR bio[™]. If using another electrophoresis system, these instructions may need to be adjusted according to the manufacturer's instructions.

- **1.** Place the gel tray containing your gel in the buffer chamber
 - Ensure that the clear buffer chamber is inside the blueGel[™] electrophoresis system.
 - The wells of the gel should be on the same side as the negative electrode, away from the power button.

2. Add 30 ml of 1X TBE electrophoresis buffer

- The buffer should just cover the gel and fill the wells.
- Ensure that there are no air bubbles in the wells (shake the gel gently if bubbles need to be dislodged).

3. Load samples onto the gel in the following sequence

- Lane 1: 10 µl DNA Ladder
- Lane 2: 15 µl Student 1 PCR product
- Lane 3: 15 μl Student 2 PCR product
- Lane 4: 15 µl Student 3 PCR product
- Lane 5: 15 μ l Student 4 PCR product

<u>Note</u>: Change pipette tips between samples to prevent contamination.



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4. Place the orange cover on the blueGel[™] electrophoresis system

- To prevent fogging, make sure that ClearView[™] spray has been evenly applied to the inside of the orange cover.
- Match the positive and negative electrode signs on the orange lid with the corresponding positive and negative signs on the blue base.
- The electrodes of the lid should be aligned with the metal leads on the base.
- The orange lid should sit flush with the blue base using littl force.

5. Press the "Run" () button

• Check that the green light beside the power button remains illuminated.

6. Conduct electrophoresis for 35-45 minutes

- Note: Check the gel every 10 minutes to monitor sample migration.
- Longer electrophoresis times will result in better size resolution. However, if run too long, small DNA fragments can run off the end of the gel or lose fluorescence.



Student's Guide





Chronotype questionnaire

1. While your gel is running complete the chronotype questionnaire on page 27.

Gel electrophoresis - Visualizing results

- Press the "light bulb" (★) button to turn on the blueGel™ transilluminator
 - For best viewing, dim lights or use Fold-a-View[™] photo documentation hood with a smartphone camera.
 - Gels may be viewed at the end of the run or periodically throughout the run.
 - If the image appears hazy, wipe off the inside of the orange cover and reapply ClearView[™] spray.
- 2. Ensure that there is sufficient band separation in the 300-500 bp range
 - Run the gel longer if needed to increase resolution.
- **3.** Document your results
 - Place Fold-a-View[™] photo documentation hood on the blueGel[™] electrophoresis system to take a picture with a smartphone or other digital camera.
 - Compare the bands from the DNA samples to the ladder to obtain size estimates.









6. Study questions

*Teachers may email team@miniPCR.com to request answer key.

1. What are some physiological processes that are controlled by your circadian clock?

2. People often have to get up earlier in the morning on weekdays than they do on weekends. Because of this, people often go to bed early on weeknights, but stay up late on the weekends. Based on what you have learned about circadian clocks, what do you think about having a different schedule for different times in the week?

3. People who work night shifts are often diagnosed with what is known as "shift work disorder". The disorder is characterized by being excessively sleepy when trying to be awake (while working late at night) and not being able to sleep when trying to (during the day). This is despite the fact that such workers will often try to keep the same schedule for long periods of time. Why might it be difficult to switch your circadian rhythm to be awake at night and asleep during the day even when you are attempting to get the same total amount of sleep as normal?

4. The circadian clock is described as a "transcription-translation negative feedback loops". What is a negative feedback loop? Can you describe a transcription-translation negative feedback loop in simple terms?



5. What are the two alleles being discussed in this lab? How do they differ genetically?

6. What are some reasons that having an allele that shows an association with a certain trait may not mean that a person actually displays that trait?

7. Is being a "morning person" or an "evening person" solely dependent on your circadian clock?

8. Reverse genetics uses techniques such as knocking out a gene (turning it off) to learn what effects that has on the organism. What might be some advantages of doing association studies over reverse genetic techniques? What might the limitations be?



Post Lab Questions

- **1.** According to the questionnaire, are you an "evening type", "morning type", or "intermediate type"? Does your result match how you normally think of yourself?
- **2.** What is your genotype and, assuming that the genetic association holds true, what does this suggest about your expected phenotype?
- 3. What are some reasons your genotype and perceived phenotype may not have matched?

4. Now look at your class data. What is the average morning-eveningness score for students who are 4/4 homozygotes, 4/5 heterozygotes, and 5/5 homozygotes? Why is it more important to look at group averages than individual scores?

Let's now look at class data slightly differently.

- 5. How many total 4-repeat alleles were there in your class data?
 - Remember that 4/4 homozygotes each have 2 alleles. 4/5 heterozygotes will have only one.



- 6. What percentage of the total alleles for each phenotype 4-repeat alleles?
- 7. How many total 5-repeat alleles were there in your class data?
 - Remember that 5/5 homozygotes each have 2 alleles. 4/5 heterozygotes will have only one.

8. What percentage of the total alleles for each phenotype 4-repeat alleles?

9. Compare the two sets of percentages. Do you see any trend from the data?

- **10.** Does your class data show a possible association between either *Per3* allele and morning or evening preference?
- **11.** Why would you need to use large sample sizes and a statistical test to establish whether an association is real or not?



Data Analysis

Create a bar graph of your class data. For each chronotype, plot how many of each genotype were present in your class. (Each chronotype should have three bars.)

	■ 4/4	■ 4/5	5/5		
Morning	Int	termediate		Evening	

Create a graph showing the average Morning-Eveningness Score for each genotype



7. Morning-Eveningess Questionnaire6

This self-assessment questionnaire to determine your circadian rhythm *chronotype* can be carried out during the PCR or gel electrophoresis runs.

Name (or sample number): ______ Date: ______

For each question, please select the answer that best describes you by circling the point value that best indicates how you have felt in recent weeks.

1. Approximately what time would you get up if you were entirely free to plan your day?

[5] 5:00 AM-6:30 AM (05:00-06:30 h
[4] 6:30 AM-7:45 AM (06:30-07:45 h)
[3] 7:45 AM-9:45 AM (07:45-09:45 h)
[2] 9:45 AM-11:00 AM (09:45-11:00 h)
[1] 11:00 AM-12 noon (11:00-12:00 h)

2. Approximately what time would you go to bed if you were entirely free to plan your evening?

- [5] 8:00 PM-9:00 PM (20:00-21:00 h)
- [4] 9:00 PM-10:15 PM (21:00-22:15 h)
- [3] 10:15 PM-12:30 AM (22:15-00:30 h)
- [2] 12:30 AM-1:45 AM (00:30-01:45 h)
- [1] 1:45 AM-3:00 AM (01:45-03:00 h)

3. If you usually have to get up at a specific time in the morning, how much do you depend on an alarm clock?

- [4] Not at all
- [3] Slightly
- [2] Somewhat
- [1] Very much

4. How easy do you find it to get up in the morning (when you are not awakened unexpectedly)?

- [1] Very difficult
- [2] Somewhat difficult
- [3] Fairly easy
- [4] Very easy

5. How alert do you feel during the first half hour after you wake up in the morning?

- [1] Not at all alert
- [2] Slightly alert



- [3] Fairly alert
- [4] Very alert

6. How hungry do you feel during the first half hour after you wake up?

- [1] Not at all hungry
- [2] Slightly hungry
- [3] Fairly hungry
- [4] Very hungry

7. During the first half hour after you wake up in the morning, how do you feel?

- [1] Very tired
- [2] Fairly tired
- [3] Fairly refreshed
- [4] Very refreshed

8. If you had no commitments the next day, what time would you go to bed compared to your usual bedtime?

- [4] Seldom or never later
- [3] Less than 1 hour later
- [2] 1-2 hours later
- [1] More than 2 hours later

9. You have decided to do physical exercise. A friend suggests that you do this for one hour twice a week, and the best time for him is between 7-8 AM (07-08 h). Bearing in mind nothing but your own internal "clock," how do you think you would perform?

- [4] Would be in good form
- [3] Would be in reasonable form
- [2] Would find it difficult
- [1] Would find it very difficult

10. At approximately what time in the evening do you feel tired, and, as a result, in need of sleep?

- [5] 8:00 PM-9:00 PM (20:00-21:00 h)
- [4] 9:00 PM-10:15 PM (21:00-22:15 h)
- [3] 10:15 PM-12:45 AM (22:15-00:45 h)
- [2] 12:45 AM-2:00 AM (00:45-02:00 h)
- [1] 2:00 AM-3:00 AM (02:00-03:00 h)

11. You want to be at your peak performance for a test that you know is going to be mentally exhausting and will last two hours. You are entirely free to plan your day. Considering only your "internal clock," which one of the four testing times would you choose?

[6] 8 AM-10 AM (08-10 h) [4] 11 AM-1 PM (11-13 h)



- [2] 3 PM–5 PM (15–17 h)
- [0] 7 PM–9 PM (19–21 h)

12. If you got into bed at 11 PM (23 h), how tired would you be?

- [0] Not at all tired
- [2] A little tired
- [3] Fairly tired
- [5] Very tired

13. For some reason you have gone to bed several hours later than usual, but there is no need to get up at any particular time the next morning. Which one of the following are you most likely to do?

- [4] Will wake up at usual time, but will not fall back asleep
- [3] Will wake up at usual time and will doze thereafter
- [2] Will wake up at usual time, but will fall asleep again
- [1] Will not wake up until later than usual

14. One night you have to remain awake between 4-6 AM (04-06 h) in order to carry out a night watch. You have no time commitments the next day. Which one of the alternatives would suit you best?

- [1] Would not go to bed until the watch is over
- [2] Would take a nap before and sleep after
- [3] Would take a good sleep before and nap after
- [4] Would sleep only before the watch

15. You have two hours of hard physical work. You are entirely free to plan your day. Considering only your internal "clock," which of the following times would you choose?

- [4] 8 AM-10 AM (08-10 h)
- [3] 11 AM-1 PM (11-13 h)
- [2] 3 PM–5 PM (15–17 h)
- [1] 7 PM–9 PM (19–21 h)

16. You have decided to do physical exercise. A friend suggests that you do this for one hour twice a week. The best time for her is between 10-11 PM (22-23 h). Bearing in mind only your internal "clock," how well do you think you would perform?

- [1] Would be in good form
- [2] Would be in reasonable form
- [3] Would find it difficult
- [4] Would find it very difficult

17. Supose you can choose your own work hours. Assume that you work a five-hour day (including breaks), your job is interesting, and you are paid based on your performance. At approximately what time would you choose to begin?

[5] 5 hours starting between 4-8 AM (05-08 h)

[4] 5 hours starting between 8–9 AM (08–09 h)



- [3] 5 hours starting between 9 AM-2 PM (09-14 h)
- [2] 5 hours starting between 2–5 PM (14–17 h)
- [1] 5 hours starting between 5 PM-4 AM (17-04 h)
- 18. At approximately what time of day do you usually feel your best?
 - [5] 5–8 AM (05–08 h)
 [4] 8–10 AM (08–10 h)
 [3] 10 AM–5 PM (10–17 h)
 [2] 5–10 PM (17–22 h)
 - [1] 10 PM-5 AM (22-05 h)

19. One hears about "morning types" and "evening types." Which one of these types do you consider yourself to be?

- [6] Definitely a morning type
- [4] Rather more a morning type than an evening type
- [2] Rather more an evening type than a morning type
- [1] Definitely an evening type



INTERPRETING AND USING YOUR MORNINGNESS-EVENINGNESS SCORE

This questionnaire has 19 questions, each with a number of points. First, add up the points you circled and **enter your total morningness-eveningness score here**: _____

Scores can range from 16-86.

- Scores of 41 and below indicate "evening types."
- Scores between 42-58 indicate "intermediate types."
- Scores of 59 and above indicate "morning types."

16-30	31-41	42-58	59-69	70-86
Definite	Moderate	Intermediate	Moderate	Definite
evening	evening		morning	morning

Occasionally a person has trouble with the questionnaire. For example, some of the questions are difficult to answer if you have been on a shift work schedule, if you don't work, or if your bedtime is unusually late. Your answers may be influenced by an illness or medications you may be taking.

One way to check this is to ask whether your morning-eveningness score approximately matches the sleep onset and wake-up times listed below:

Score	16-30	31-41	42-58	59-69	70-86
Sleep onset	2.00 am-	12.45 am-	10.45 pm-	9.30 pm -	9.00 pm –
	3.00 am	2.00 am	12.45 am	10.45 pm	9.30 pm
Wake up	10.00 am -	8.30 am-	6.30 am -	5.00 am –	4.00 am –
	11.30 am	10.00 am	8.30 am	6.30 am	5.00 am

Share your data:

Help towards a better understanding of this postulated genetic association.

Most phenotypes are complex traits, with multiple genetic and environmental components. Genetic associations are difficult to establish unequivocally, and association studies are notoriously hard to reproduce, especially due to limitations in sample size. By compiling anonymized data across our classrooms, we can contribute to a deeper understanding of this postulated association between *Per3* alleles and sleep preferences.

If you're interested in contributing your classroom's anonymized data to this ongoing investigation, please contact: <u>team@minipcr.com</u>.

We are happy to provide guidance on data gathering, anonymization, and sharing (in the format provided here: <u>https://www.minipcr.com/wp-content/uploads/sleep-lab-data.xlsx</u>).

8. Extension activity: Hypothesized mechanism⁵:



kinase 1 (CK1) E phosphorylation are indicated above by filled circles (the consensus CK1E motif is Sp/Tp-X₁₋₃-<u>S/T</u>, where the lead serine or threonine must be prephosphorylated, X is 1-3 nonspecific spacing residues, and the target serine or threonine is underlined).

As a reminder, CLOCK and BMAL1 are transcription factors activating the expression of circadian clock genes, including *Period* (PER) and *Cryptochrome* (CRY). PER and CRY proteins are in turn repressors of CLOCK/BMAL1 activity, shutting down their own activation.

PER and CRY proteins accumulate throughout the circadian day, peaking in the early night, when they move into the nucleus and turn off their own transcription by blocking Clock and Bmal1 (see p. 10 inset for a schematic model).

PER proteins undergo phosphorylation by two *kinases*, enzymes that phosphorylate (covalently link phosphate groups) other proteins as part of signaling processes within the cell. Kinases in the circadian feedback loop are Casein kinase 1 δ and ϵ (CK1 δ and CK1 ϵ), and it is known that by phosphorylating PER and CRY proteins they target them for degradation, which results in decreased PER and CRY protein levels in the early circadian day. With low PER and CRY levels, repression of Clock/BMAL1 is lifted, and transcription can begin again. Thus, a new circadian cycle starts early in the prospective day.

Hypothesizing a model where PERIOD3 undergoes phosphorylation in the same manner described above, and that the 5-repeat VNTR <u>adds</u> new phosphorylation sites to the PER3 protein, **ask students to draw a model for how the** *Per3^{5/5}* **polymorphism may result in a morning lark phenotype**.

⁵ Figure from Archer SN, et al. "A length polymorphism in the circadian clock gene Per3 is linked to delayed sleep phase syndrome and extreme diurnal preference." Sleep. 2003 Jun 15;26(4):413-5.

9. Expected results



Results represent a 45 min run using a blueGel[™]

Interpretation

The larger ~400 bp band represents the *per3* 5 allele and the smaller ~350 bp band represents the *per3* 4 allele. Make sure you run your gel long enough to have clear separation of the bands

- per3 5,5 homozygotes will have only the larger ~400 bp band
- *per3* 4,5 heterozygotes will have both the ~400 bp band and the ~350 bp band
- *per3* 4,4 homozygotes will have only the smaller ~350 bp band

A study found the following distribution of genotypes in Norwegian university students⁶:

0	192 per3 ⁴⁻⁴	(44%)
0	191 per3 ⁴⁻⁵	(44%)
0	49 per3 ⁵⁻⁵	(11%)



Can you determine the frequency of the *per3*-4 and -5 alleles in this sample? ...And in your class?

⁶ Osland TM, *et al.*, Association study of a variable-number tandem repeat polymorphism in the clock gene PERIOD3 and chronotype in Norwegian university students. Chronobiol Int. 2011 Nov;28(9):764-70.

10. References and teaching resources

- 1. "Circadian Clock." Wikipedia.
- 2. "<u>Clockwork Genes. Discoveries in Biological Time</u>." HHMI Biointeractive.
- **3.** "<u>The Mammalian Molecular Clock Model</u>." *HHMI Biointeractive*.
- **4.** Archer, SN et. al. "<u>A length polymorphism in the circadian clock gene Per3 is linked to delayed</u> <u>sleep phase syndrome and extreme diurnal preference</u>." *Sleep.* 2003 Jun 15;26(4):413-5.
- Ebisawa, T., Uchiyama, M., Kajimura, N., Mishima, K., Kamei, Y., Katoh, M., Watanabe, T., Sekimoto, M., Shibui, K., Kim, K., et al. "Association of structural polymorphisms in the human *period3* gene with delayed sleep phase syndrome". *EMBO Reports*. 2001. 2, 342–346. 10.
- **6.** Ko, Caroline and Takahasi, Joseph. "<u>Molecular components of the mammalian circadian clock</u>." *Human Molecular Genetics.*
- Osland TM, et al., "Association study of a variable-number tandem repeat polymorphism in the clock gene PERIOD3 and chronotype in Norwegian university students." *Chronobiol Int.* 2011 Nov;28(9):764-70.
- 8. Viola, AU et. al. "<u>Per3 polymorphism predicts sleep structure and waking performance.</u>" *Current Biology.* 2007 Apr 3;17(7):613-8. Epub 2007 Mar 8

11. Learning goals and skills developed

Student Learning Goals:

- Understand that PCR is a technique for amplifying specific parts of the genome
- Learn about the application of DNA analysis in genotype identification and in the study of inheritance, human genetics, DNA polymorphisms, and genetic diversity
- Extract meaningful information from genetic polymorphisms, particularly variable number tandem repeats (VNTRs)
- Explore the circadian regulation of behavior and its role in sleep homeostasis
- Construct models of circadian transcriptional/translational feedback loops
- Understand the concept of genetic association through data analysis

Scientific Inquiry Skills:

- Create hypotheses and make prediction about results
- Compare experimental results to their predictions
- Generate graphics and tables to present their results
- Perform statistical analyses on aggregate genetic data
- Make conclusions based on their experimental results about their hypothesis

Molecular Biology Skills:

- Micropipetting
- DNA extraction (from cheek cells)
- PCR set-up and execution
- Preparation of agarose gels
- Agarose gel electrophoresis
- Staining, visualization, and molecular weight analysis of DNA fragments



12. Standards Alignment

Next Generation Science Standards - Students will be able to...

- HS-LS1-1 Construct an explanation based on evidence for how the structure of DNA determines the structure of proteins which carry out the essential functions of life through systems of specialized cells.
- HS-LS3-1 Ask questions to clarify relationships about the role of DNA and chromosomes in coding the instructions for characteristic traits passed from parents to offspring.
- HS-LS3-2 Make and defend a claim based on evidence that inheritable genetic variations may result from (1) new genetic combinations through meiosis, (2) viable errors occurring during replication, and/or (3) mutations caused by environmental factors.
- HS-LS3-3 Apply concepts of statistics and probability to explain the variation and distribution of expressed traits in a population.
- HS-LS4-1 Communicate scientific information that common ancestry and biological evolution are supported by multiple lines of empirical evidence.

Common Core English Language Arts Standards - Students will be able to...

- RST.11-12.1 Cite specific textual evidence to support analysis of science and technical texts, attending to important distinctions the author makes and to any gaps or inconsistencies in the account.
- WHST.9-12.2 Write informative/explanatory texts, including the narration of historical events, scientific procedures/ experiments, or technical processes.
- WHST.9-12.9 Draw evidence from informational texts to support analysis, reflection, and research.
- SL.11-12.5 Make strategic use of digital media (e.g., textual, graphical, audio, visual, and interactive elements) in presentations to enhance understanding of findings, reasoning, and evidence and to add interest.

AP Biology Big Idea 3: Living systems store, retrieve, transmit and respond to information essential to life processes

- 3.A.1: DNA, and in some cases RNA, is the primary source of heritable information.
- 3.A.2: In eukaryotes, heritable information is passed to the next generation via processes that include the cell cycle and mitosis or meiosis plus fertilization.
- 3.A.3: The chromosomal basis of inheritance provides an understanding of the pattern of passage(transmission) of genes from parent to offspring.
- 3.A.4 The inheritance pattern of many traits cannot be explained by simple Mendelian genetics.
- 3.C.1 Changes in genotype can result in changes in phenotype.

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Ordering information

To order miniPCR[®] Sleep Lab kits, you can:



Call (781)-990-8PCR



] email us at orders@minipcr.com



visit <u>https://www.minipcr.com</u>

miniPCR[®] Sleep Lab (catalog no. KT-1005-01) contains the following reagents:

- 5X EZ PCR Master Mix, Load Ready™
- Sleep Lab Primers
- X-Tract[™] DNA Extraction Buffer
- 100 bp DNA Ladder, Load Ready™

Materials are sufficient for 32 students

All components should be kept frozen at -20°C for long-term storage

Reagents must be used within 12 months of shipment

Other supplies and reagents needed

- Agarose (electrophoresis grade)
- Fluorescent DNA stain (e.g., SeeGreen[™] or GelGreen[®])
- Gel electrophoresis buffer (e.g., 1X TBE)
- Distilled or deionized water (to dilute TBE buffer concentrate)
- Toothpicks

Note: Agarose, DNA stain, and TBE buffer are available at minipcr.com as part of the Learning Lab Companion Kit (KT-1510-01)

Instructor's Guide

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14. About miniPCR Learning Labs

This Learning Lab was developed by miniPCR[™] in an effort to make molecular biology and genetics more approachable and accessible.

We believe that there is no replacement for hands-on experimentation in the science learning process. Our goal is to augment everyone's love of scientific inquiry and STEM. We also believe, based on our direct involvement working in educational settings, that it is possible for these experiences to have a positive impact in students' lives. Our goal is to increase everyone's love of DNA science, scientific inquiry, and STEM. We develop Learning Labs to help achieve these goals, working closely with educators, students, academic researchers, and others committed to science education.

This lab was the result of a project spearheaded at miniPCR by Catherine Smilow, a student at Harvard College passionate about human genetics. Sebastian Kraves, miniPCR co-founder, also happened to have done his PhD on the molecular control of circadian behavior. The two loved the idea of a Learning Lab that could link genes to behavior through the circadian clock, while also inviting students to explore advanced topics such as genetic associations. For the record, Katie is heterozygous for the *Per3* locus, while Sebastian's genotype is that expected for a night owl.

Starting on a modest scale working with AP Biology Summer Institute educators, miniPCR[™] Learning Labs in human genetics are gaining adoption, and their use is growing rapidly through academic and outreach collaborations.

Authors: Ezequiel Alvarez Saavedra, Ph.D., Sebastian Kraves, Ph.D., Catherine Smilow and Bruce Bryan.