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Laboratory Exercise

Human Xq28 Inversion Polymorphism: From Sex Linkage to Genomics - A Genetic Mother Lode

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Abstract

An inversion polymorphism of the filamin and emerin genes at the tip of the long arm of the human X-chromosome serves as the basis of an investigative laboratory in which students learn something new about their own genomes. Long, nearly identical inverted repeats flanking the filamin and emerin genes illustrate how repetitive elements can lead to alterations in genome structure (inversions) through nonallelic homologous recombination. The near identity of the inverted repeats is an example of concerted evolution through gene conversion. While the laboratory in its entirety is designed for college level genetics courses, portions of the laboratory are appropriate for courses at other levels. Because the polymorphism is on the X-chromosome, the laboratory can be used in introductory biology courses to enhance understanding of sex-linkage and to test for Hardy-Weinberg equilibrium in females. More advanced topics, such as chromosome interference, the molecular model for recombination, and inversion heterozygosity suppression of recombination can be explored in upper-level genetics and evolution courses. DNA isolation, restriction digests, ligation, long PCR, and iPCR provide experience with techniques in molecular biology. This investigative laboratory weaves together topics stretching from molecular genetics to cytogenetics and sex-linkage, population genetics and evolutionary genetics. © 2016 by The International Union of Biochemistry and Molecular Biology, 44:191–201, 2016.

A Genetic Mother Lode

On the long arm of the X-chromosome (Xq28) lie two long inverted repeats (LIRs, roughly 11.3 kb in length, and greater than 99% identical) which flank two genes—emerin and filamin [1]. Small *et al.* determined a patient's Emery-Dreifuss muscular dystrophy (EDMD) was due to a complete emerin deletion [1]. While analyzing the Xq28 region, Small discovered a frequent inversion polymorphism in other individuals of European ancestry—the order of the aforementioned genes is variable [1]. The plus (+) orientation reads (cen-LIR-*FLNA*-*EMD*-LIR-tel) and the minus (−) orientation reads (cen-LIR-*EMD*-*FLNA*-LIR-tel) as diagrammed in Fig. 1. This polymorphism is not linked to any phenotypic effects.

The idea for a teaching laboratory emerged upon seeing a Southern blot autoradiograph of BglII digests which showed single-banded female homozygotes, double-banded female heterozygotes and single-banded male hemizygotes [1]. Since the radioactive probe necessary to detect the fragments of single-copy genes on Southern blots is not appropriate for use in a teaching laboratory, we have developed two PCR techniques—long PCR and inverse PCR (iPCR)—to determine an individual's gene arrangements. These PCR assays can be used as a vehicle for teaching sex linkage, calculating genotypic frequencies for the two gene arrangements in the student population and checking for Hardy-Weinberg equilibrium in the female portion of the sample. Additional work on this polymorphism provides the opportunity to discuss inverted repeats as inversion breakpoint hotspots, inversions as suppressors of recombination, gene conversion leading to concerted evolution and chromosome interference in double crossovers.

In 2007, Caceres et al. examined the *FLNA-EMD* region in 16 species of eutherian mammals; each species studied has long inverted repeats (LIRs) flanking the genes, the presence of which indicates that the duplication event resulting in the LIRs occurred more than 100 million years ago [2]. The LIRs share high sequence identity within species (most greater than 99%!), but low sequence identity between species. The LIRs are evolving together within a species while evolving away from one another between species, a process known as concerted evolution, which can be explained by the "sex and the single X" phenomenon, as outlined below.

In prophase I of oogenesis, all 23 pairs of homologous chromosomes pair up along their lengths due to sequence similarity. While the autosomes can pair up normally in spermatogenesis, the X and Y sex chromosomes can only pair in the pseudoautosomal regions at the tips of their arms leaving the X still looking for a pairing partner for most of its length. Not finding another X with which to pair, the inverted repeats pair intrachromosomally, creating the opportunity for nonallelic homologous recombination (NAHR). This recombination can take two forms—crossing over to change one gene arrangement to the other (i.e. inversion) and gene conversion to maintain inverted repeat identity. Figure 2 illustrates the pairing of the inverted repeats in a plus X chromosome with an inset showing the two Holliday junctions and heteroduplex DNA which set the stage for crossing over and gene conversion respectively. Crossing over between the paired inverted repeats will invert the *FLNA-EMD* region to produce a minus X chromosome. Gene conversion will either fix or eliminate
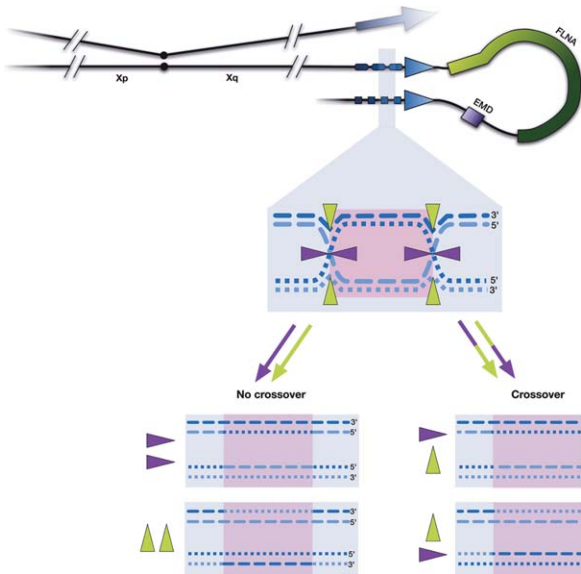

FIG 2 *Nonallelic homologous recombination within a single chromatid resulting from intrachromosomal pairing of the inverted repeats. The inset shows double stranded DNA forming two Holliday junctions bracketing a heteroduplex region—the large ovals and the small squares represent the double-stranded DNA molecules in the proximal and distal IRs, respectively. Though Holliday junctions permit crossing over, the resolution of the junctions determines whether or not crossing over occurs. If the two junctions are resolved the same way (endonuclease cuts at the four green arrows or the four purple arrows) no crossover results but if they are resolved differently (one junction cut at the green arrows and the other at the purple arrows) a crossover results. Mismatched base pairs in the heteroduplex region may lead to gene conversion which keeps the IRs similar.*
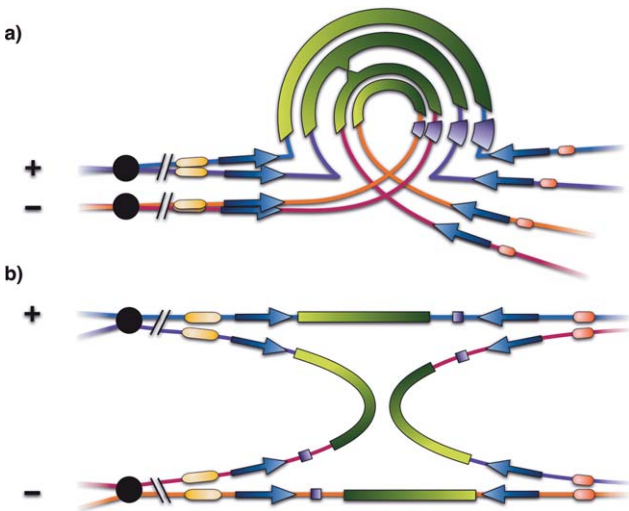

FIG 3 *A prophase I inversion loop in a +//− heterozygote is diagramed in (a) with a crossover in FLNA. The duplication- and deletion-containing dicentric anaphase bridge (two yellow ovals and no red oval) and acentric fragment (two red ovals and no yellow oval) resulting from the crossover are shown in (b) along with the two parental chromatids not involved in the crossover.*
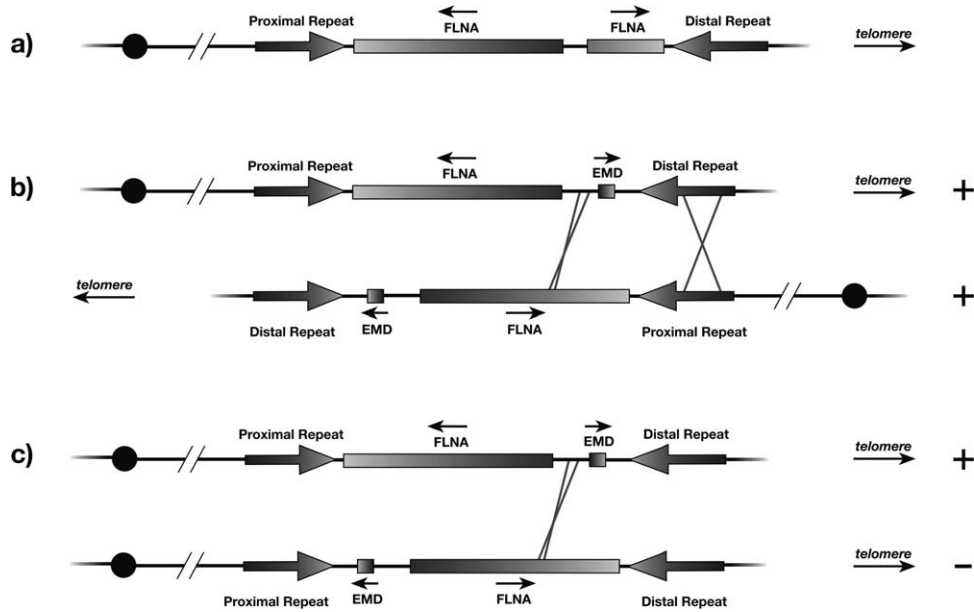
FIG 4 The EMD-deficient and FLNA-partially-duplicated Emery-Dreifuss X-chromosome is shown in (a). The Small et al. [1] model explaining the origin of this chromosome (a) in a homozygous +//+ mother is shown in (b). An alternative origin in a heterozygous +//− mother is shown in (c). There is sequence data supporting the unequal crossover required in both models but the second crossover in (b) is purely hypothetical. Model (c) requires only that a rare unequal crossover resulted from misalignment due to inversion heterozygosity while (b) requires similar misalignment but in a +//+ homozygote creating a dicentric chromatid that must be resolved by a hypothetical second crossover within the inverted repeats. This hypothetical crossover is highly unlikely due to chromosome interference.
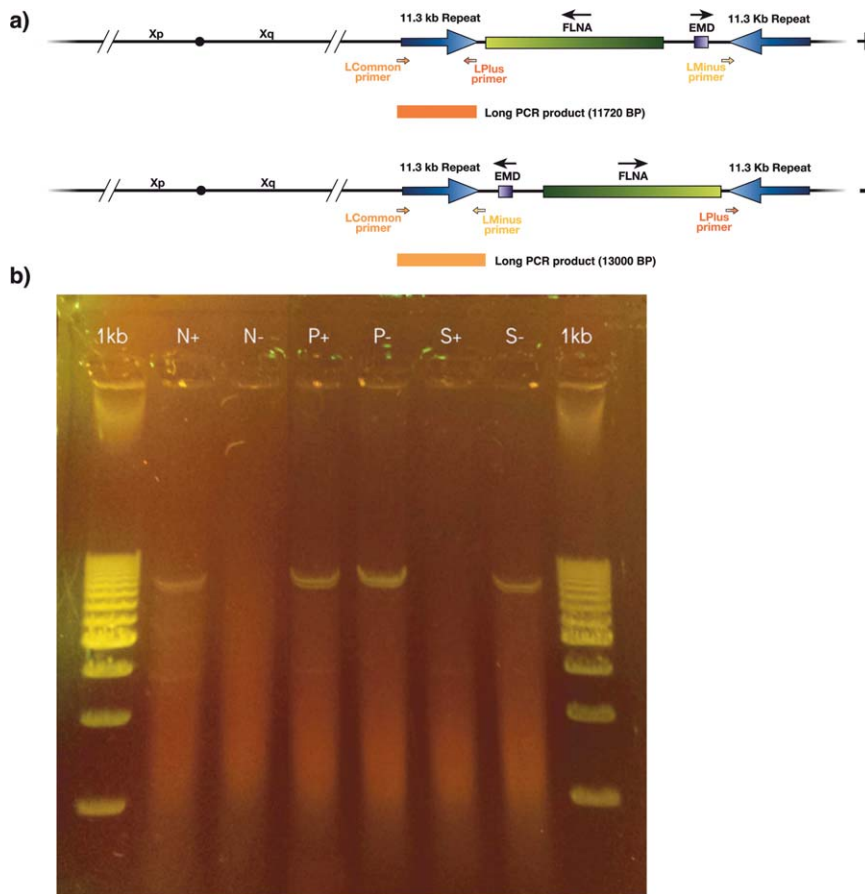
FIG 5 Primer design for the presence-absence long PCR assay is shown in (a). The + reaction uses LCommon and LPlus primers, while the − uses LCommon and LMinus. (Note that not all components (e.g. primers) are drawn to scale.) The approximately 12 kb and 13 kb PCR products are shown in (b): the presence of a band in N+ but the absence of a band in N− indicates that the female individual N is a homozygote +//+; the presence of a band in P+ and in P− indicates that the female individual P is a heterozygote +//−; the absence of a band in S+ but the presence of a band in S− indicates that the male individual S is a hemizygote −/y. Lanes designated 1 kb are 1 kb ladder.
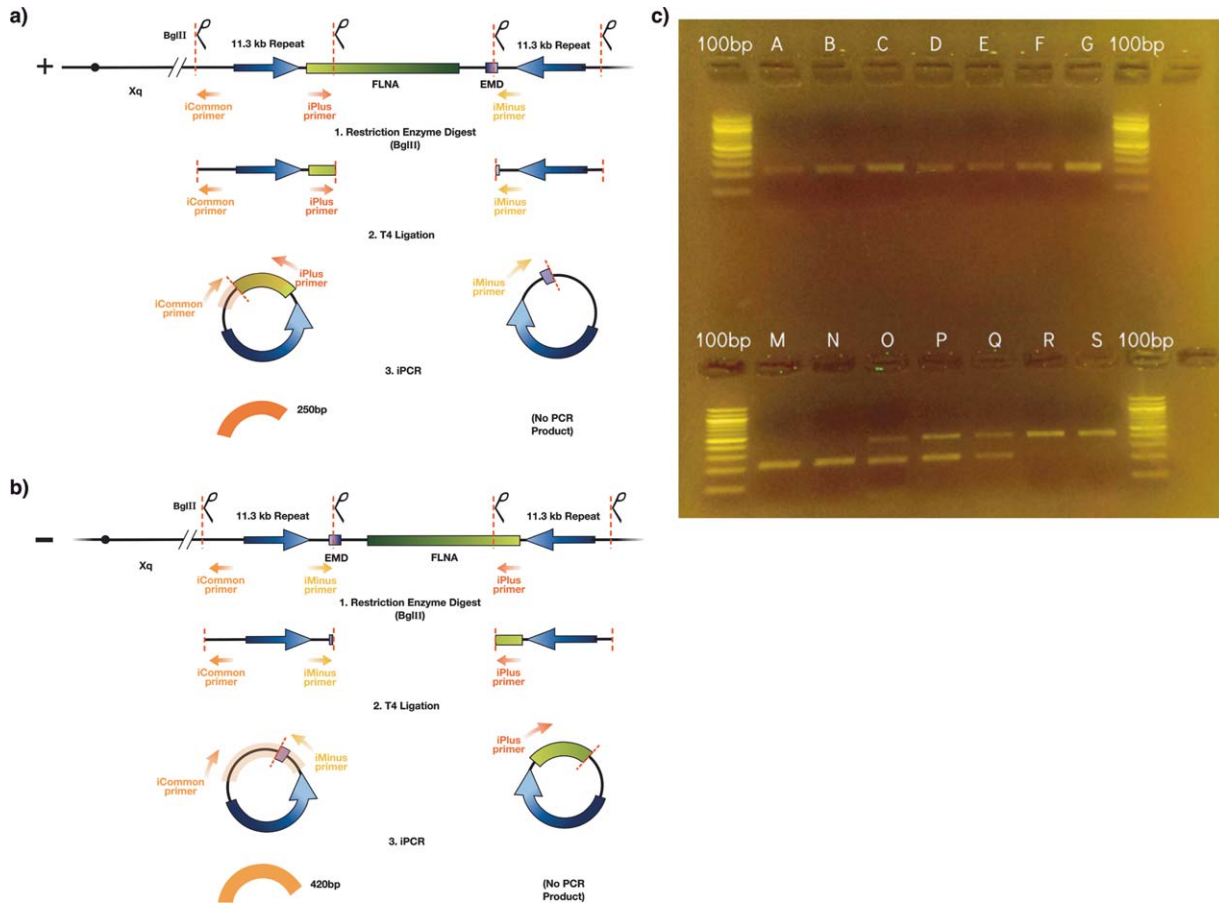
The iPCR assay including the restriction digest, ligation, and primer design for the inverse PCR is shown for the + reaction in (a) and the minus reaction in (b). (Note that not all components (e.g. primers) are drawn to scale.) The final PCR product from step 3 is shown in (c): lanes A and B are hemizygote +/y males, lanes C-G, M and N are homozygote +//+ females, lanes O-Q are heterozygote +//− females, lane R is a homozygote −//− female and lane S is a hemizygote −/y male. Lanes designated 100 bp are 100 bp ladder.

any mutation resulting in mismatched base pairs within the heteroduplex and thereby maintain inverted repeat sequence identity (concerted evolution) while allowing for repeats in different species to evolve away from one another. Thinking back to the Caceres article mentioned above, it seems unlikely that species exhibiting these two genes between two long IRs would be monomorphic for this inversion if our proposed mechanism is correct—NAHR should be changing the order of genes at some rate. The fact that those authors only saw one orientation or the other may be due to sample size or sampling from one population.

The nonallelic homologous recombination described above is probably less common in oogenesis where each X chromosome has a homolog with which to pair. Oogenesis does provide the opportunity for crossing over to recombine mutations that occur on the two X-chromosomes. Inversion heterozygosity results in suppression of recombination which leads to a nonrandom association of alleles with the different gene arrangements called linkage disequilibrium. If crossing over occurs within a paracentric inversion (like the inversion being explored here), the result is that all recombinant chromatids contain duplications and deletions and are either dicentric anaphase bridges or acentric fragments—the recombinant chromatids do not segregate to functional gametes/viable offspring (Fig. 3).

Small et al. (1997) explained the deletion of EMD and the partial duplication of FLNA by a double crossover event in a plus/plus homozygote [1]. Their model involved an unequal crossover event (rare) in misaligned chromosomes coupled with an adjacent crossover within a repeat region (also rare). There is sequence data supporting the unequal crossover resulting in the EMD deletion and FLNA partial duplication, while the second crossover in the Small et al. model is hypothetical but necessary to avoid a dicentric chromosome. This second, hypothetical crossover in close proximity (within 20 kb) to the unequal crossover is highly unlikely due to chromosome interference. A group studying mouse chromosome 1 found complete inhibition of a second crossover event over a distance of approximately 40 million base pairs [3]. A simpler model involving the same abnormal pairing within the inverted region during oogenesis in a plus/minus heterozygote eliminates the need for this second hypothetical crossover, both models are shown

in Fig. 4. The genotype of the dystrophic patient's mother was not determined thus it is impossible to prove which model is the correct one, but these two explanations provide an opportunity to discuss the relationship between inversions, crossing over, and chromosome interference.

Inverted repeats (IRs) are a common feature of the human genome; one study identified more than 22,624 highly similar IRs ranging in size from 25 bp to >100 kb [4]. That study found that of 96 IRs greater than 8 kb, nearly 25% occur on the X-chromosome and compared to IRs on autosomes, the X-linked IRs are three times more likely to share at least 99% sequence identity. The Y-chromosome also contains a disproportionate share of the large IRs. While the Xq28 IRs investigated in this laboratory exercise do not contain genes, many of the large IRs do contain genes and those genes are frequently testes-specific. The open questions arising from these observations create an opportunity for class discussion: why are the large nearly identical IRs concentrated on the sex chromosomes? Why do these IRs often contain testes specific genes? Is there some advantage of having two copies of these testes specific, sex-linked genes in males?

In the following protocols section we provide two approaches for genotyping students for the Xq28 inversion polymorphism both of which are demanding techniques and care must be taken not to shear the DNA. The first is a long PCR assay (illustrated in Fig. 5) producing two amplicons—the plus amplicon length is 11,700 bp and the minus amplicon length is 13,000 bp. The second method is a multiplex iPCR assay (illustrated in Fig. 6) which involves a restriction digest, ligation and a short PCR producing two amplicons—the plus amplicon is roughly 250 bp, the minus amplicon is roughly 420 bp.

The sample data presented below contain DNA samples extracted by students in an introductory biology course; the genotyping assays were initially performed by the students in class and those results were reproduced by author C.S. Kirby for the purposes of this paper.

Acknowledgments

Protocols

Protocol 1: DNA Extraction

Epicentre Illumina Masterpure DNA Kit cat # MC85200.
Epicentre Illumina Catch-All Sample Collection Swab Soft Pack cat # QEC89100.

1) Scrape left cheek 30 times with one sterile swab, rolling, and twirling.

2) Repeat step (1) with right cheek.
3) Twirl swabs in one clear eppie tube labeled with your initials of 300 µL cell lysis solution + 1 µL PK solution. Be sure to squeeze the swab into the edge of the tube to remove all liquid. Finger flick tube.
4) Incubate at 56°C for 15 min, finger flicking after every 5 min.
5) Let tubes cool to 37°C. Add 1 µL RNase A solution. Finger flick.
6) Incubate at 37°C for 30 min.
7) Let sit on ice for 5 min.
8) Add 150 µL MPC Protein Precipitation Reagent. Finger flick.
9) Centrifuge at max speed for 10 min.
10) Transfer the supernatant to a new clear eppie tube labeled with your initials and "gDNA." Discard pellet.
11) Add 500 µL **cold** isopropanol to the supernatant. Invert gently 30 to 40 times until you see a floating swirl of DNA.
12) Centrifuge at max speed for 10 min. You should see a pellet. Pour off isopropanol. Be careful not to pour off the pellet.
13) Wash with 500 µL 70% **cold** ethanol twice. If pellet dislodges, centrifuge again.
14) Centrifuge at max speed for 1 to 2 min. Remove all excess ethanol with a pipette.Repeat ethanol removal step if necessary.
15) Leave tube open at 4°C to evaporate ethanol. Evaporate for 30 min—24 h depending upon the amount of residual ethanol.
16) Once ethanol is completely evaporated, resuspend pellet overnight at 4°C in 100 µL TE buffer.
17) The DNA will not go into solution immediately. DNA should work for long PCR for up to 6 months when stored at 4°C. For longer storage, −20°C is suitable, but avoid freeze-thawing as that will shear DNA.

Protocol 2: LongAmp PCR Assay
LongAmp PCR Reaction

1. For each sample to be amplified, a master mix is made as follows:
   a. Plus Reaction (pink tube):
      - 5.5 µLddH$_2$0
      - 12.5 µL 2× MasterMix (Promega Go Taq Long Polymerase, cat # M4021)
      - 1 µL LCommon Primer (10 µM) (CGA TGG GGT CTA ACA GGT GTG ATG C)
      - 1 µL LPlus Primer (10 µM) (GCA AGG CTC TCG GGG GCA ACG)
   b. Minus Reaction (blue tube):
      - 5.5 µLddH$_2$0
      - 12.5 µL 2× MasterMix (Promega Go Taq Long Polymerase, cat # M4021)

- 1 μL LCommon Primer (10 μM) (CGA TGG GGT CTA ACA GGT GTG ATG C)
- 1 μL LMinus Primer (10 μM) (CGT TTC AGG GCT CCT CAG GGT TCT GC)

2. Aliquot 20 μL of the master mix per student for both the plus and minus reactions. Each student will add 5 μL of their DNA from Protocol 1 to both tubes. The PCR tubes containing 25 μL will be put into the PCR machine and will follow the program as listed:

- 1 cycle of
    - ○ 98°C   2 min
- 30 cycles of
    - ○ 98°C   30 s
    - ○ 67°C   15 min
- 1 cycle of
    - ○ 67°C   15 min
    - ○ 4°C   until collected

Gel Electrophoresis of LongAmp PCR Product

1. Pour a 0.8% agarose gel in 1x TBE Buffer with 35 μL SYBR Safe per 50 mL of agarose. (Certified Molecular Biology Agarose Bio-Rad cat # 161-3100; 10× TBE Buffer Bio-Rad cat # 161-0770EDU; SYBR Safe Life Technologies cat # S33102)
2. Students will add 5 μL of 5X Blue Juice (NEB 6X Tracking Dye cat #B7021S) to each of their two tubes containing PCR product.
3. Students will load as much of their product into a well as will fit ~25 μL from each tube: plus (pink) on the left, minus (blue) on the right. We read gels from left to right. Be careful not to allow any product to flow out and over into adjacent lanes as this will impair genotyping.
4. Load 8 μL of 1 kb Molecular Ruler as a reference in the outer two lanes. (Bio-Rad cat # 170-8355)
5. Using a gel electrophoresis apparatus, run the gel at ~60 V for ~2 h.
6. Visualize using a blue-light or UV transilluminator.

Protocol 3: iPCR Assay

Primers and general assay design outlined in Aguado *et al.* [5] and refined for this laboratory exercise.

Restriction Enzyme Digest Reaction

1. For each sample to be digested, a master mix is made as follows:

- 5 μL BglII Buffer (NEB cat # R0144S)
- 40 μL H$_2$O
- 1 μL BglII Enzyme (NEB cat # R0144S)

2. Dispense the master mixes in one 46 μL aliquot for each student. Each student will add 4 μ of their DNA from their clear eppie tube from Protocol 1 to their green PCR tube.

The PCR tubes containing 50 μL will be put into the PCR machine and will follow the program as listed:

- 37°C   17 h
- Add 0.5 μL enzyme to each tube (this is very important—see Setup sheet)
- 37°C   2 h
- 85°C   20 min (to inactivate enzyme)
- 4°C   until collected

Ligation Reaction

Dispense the master mix in one 34 μL aliquot per student. Each student will add 16 μL of their restriction enzyme digest reaction from their green tube from Restriction Enzyme Digest Reaction section to their yellow PCR tube. The PCR tubes containing 50 μL will be put into the PCR machine and will follow the program as listed:

- 16°C   19 h
- 25°C   3 h
- 65°C   10 min (to inactivate enzyme)
- 4°C   until collected

iPCR Reaction

1. For each sample to be amplified, a master mix is made as follows:

- 5.5 μLH$_2$O
- 12.5 μL AmpliTaq Gold 360 2× Master Mix (Life Technologies cat# 4398881)
- 1 μL iCommon Primer (10 μM) (TCA TCA CGC GAA AAA CAG AG)
- 0.5 μL iPlus Primer (10 μM) (CAC ATA AGC CAC ACC ACA GG)
- 0.5 μL iMinus Primer (10 μM) (TGC ATC CCC TCT AGT CGA A)

This iPCR protocol contains the primers and a modified version of the protocol described in Aguado et al. [5].

2. Dispense the master mix in one 20 μL aliquot per student. Each student will add 5 μL of their ligation reaction from their yellow tube from Ligation Reaction section to their orange tube. The PCR tubes containing 25 μL will be put into the PCR machine and will follow the program as listed:

- 1 cycle of
    - ○ 98°C   5 min
- 35 cycles of
    - ○ 95°C   30 sec
    - ○ 55°C   1 min
    - ○ 72°C   3.5 min
- 1 cycle of
    - ○ 72°C   7 min
    - ○ 4°C   until collected

Gel Electrophoresis of iPCR Product

1. Pour a 1.5% agarose gel in 1x TAE Buffer with 35 μL SYBR Safe per 50 mL of agarose. (Supra Sieve GPG Agarose American Bioanalytical (AmBio) cat# AB00985-00100; 50X TAE Buffer AmBio cat # AB14112-01000; Life Technologies SYBR Safe cat # S33102)

DNA extraction
2 h in lab
resuspend in TE
overnight

BglII digest
5 min in lab
Digest overnight

T4 ligation
5 min in lab
Ligate overnight

iPCR
5 min in lab
3.5 h PCR

Agarose gel
30 min setup
Hour-long gel run
10 min imaging

Conceptual
1 worksheet

iPCR assay
Five lab sessions
(only two full labs)

Long PCR
10 min in lab
PCR overnight

Agarose gel
30 min setup
2 h long gel run
10 min imaging

Long PCR assay
Three lab sessions
(only two full labs)

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2. Students will add 5 μL of 5X Blue Juice (NEB 6X Tracking Dye cat # B7021S) to their orange tube containing PCR product. Be careful not to allow any product to flow out and over into adjacent lanes as this will impair genotyping.
3. Students will load as much of their product into a well as will fit ~25 μL.
4. Load 8 μL of 100 bp ladder (NEB cat # N3231L) as a reference.
5. Using a gel electrophoresis apparatus, run the gel at 140 V for ~30 min.
6. Visualize using a blue-light or UV transilluminator.

Teaching Laboratory Exercise: Setup Sheet for Instructors

This setup sheet involves color-coding to limit the likelihood of mistakes. If your institution does not have the same colors or enough colors, I **highly** suggest that you find a different way to differentiate between tubes, such as putting a different colored dot on each tube.

DNA Extraction
To set up the DNA extraction for 16 students, you will need:

- 16 tube racks (8 if students work in pairs)
- 16 purple eppie tubes [2 mL] with lysis buffer (300 μL)
- 16 yellow eppie tubes [1.5 mL] with MPC protein precipitation reagent (150 μL)
- 16 green eppie tubes [1.5 mL] with isopropanol (500 μL)
- 16 blue eppie tubes [1.5 mL] with ethanol (1 mL)
- 16 clear eppie tubes [2 mL] empty at start [with gDNA in TE buffer at end (100 μL)]

Long PCR
To set up the long PCR assay for 16 students, you will need:

- 1 pink eppie tube [1.5 mL] (plus cocktail)

- 1 blue eppie tube [1.5 mL] (minus cocktail); 16 pink PCR tubes [0.2 mL] (plus)
- 16 blue PCR tubes [0.2 mL] (minus)
- 1 tube of Promega GoTaq Long
- 1 tube of LCommon primer
- 1 tube of LPlus primer
- 1 tube of LMinus primer

Make up the two mastermixes as follows, making enough cocktail for 17 reactions:

Plus each		(# of students + 1)	Plus cocktail
5.5 μL	H₂O	×17	93.5 μL H₂O
12.5 μL	Promega GoTaq Long	×17	212.5 μL Promega GoTaq Long
1 μL	LCommon	×17	17 μL LCommon
1 μL	LPlus	×17	17 μL LPlus
Minus each		(# of students + 1)	Minus cocktail
5.5 μL	H₂O	×17	93.5 μL H₂O
12.5 μL	Promega GoTaq Long	×17	212.5 μL Promega GoTaq Long
1 μL	LCommon	×17	17 μL LCommon
1 μL	LMinus	×17	17 μL LMinus

The instructor will then aliquot 20 μL of plus cocktail into the pink PCR tubes, and 20 μL of minus cocktail into the blue PCR tubes. The instructor should have excess cocktail

left over. Students will then add 5 μL of their DNA into each of their PCR tubes (pink and blue).

The instructor will program the thermal cycler as follows:

- 1 cycle of
  - ○ 98°C   2 min
- 30 cycles of
  - ○ 98°C   30 s
  - ○ 67°C   15 min
- 1 cycle of
  - ○ 67°C   15 min
  - ○ 4°C   until collected

Tubes should be put in the PCR machine after the initial denaturation temperature has been reached (98°C).

Agarose Gel Electrophoresis

The instructor should make up roughly 1 L of 1× TBE buffer per two gels. The TBE buffer suggested for purchase here is 10× concentrated, so for every 1 L, the instructor should mix 100 mL 10× buffer with 900 mL ddH2O, for every 2 L, 200 mL 10× buffer with 1800 mL ddH2O, etc.

The instructor should make up 50 mL of 0.8% agarose per gel: 50 mL of 1× TBE buffer with 0.4 g of agarose; once cool to the touch, add 35 μL of Sybr Safe, stir, and pour. Be sure to keep the gels away from light until they are to be used. The pre-poured gels may be stored in buffer, in a sealed container, protected from light, for up to 1 week.

Instructors may choose for students to pair up, or work in small groups, to load their gels, so the number of gels necessary per class is at the instructor's discretion. Each student will need two wells (one for the plus reaction, one for the minus).

Students will need 5 μL of 5× BJ for each lane (so 10 μL each, in total). Students may also want to practice loading their gel in spare lanes with 20 μL 1× BJ.

iPCR

To set up the iPCR BglII **digest** for 16 students, you will need:

- 1 green eppie tube [1.5 mL] (digest cocktail)
- 16 green PCR tubes [0.2 mL] (digest)
- 1 tube of BglII buffer
- 1 bottle of nuclease-free water
- 1 tube of BglII enzyme

Digest each		(# of students + 1)	Digest cocktail	
5 μL	BglII buffer	×17	85 μL	BglII buffer
40 μL	H₂O	×17	680 μL	H₂O
1 μL	BglII enzyme	×17	17 μL	BglII enzyme

The instructor will aliquot 46 μL of this digest mix to each student. The instructor should have excess cocktail left over. Each student will add 4 μL of their genomic DNA from their clear eppie tube to their green digest tube.

To set up the iPCR T4 ligation for 16 students, you will need:

- 1 yellow eppie tube [1.5 mL] (ligation cocktail)
- 16 yellow PCR tubes [0.2 mL] (ligation)
- 1 tube of T4 buffer
- 1 bottle of nuclease-free water
- 1 tube of T4 ligase

Ligation each		(# of students + 1)	Ligation cocktail	
5 μL	T4 buffer	×17	85 μL	T4 buffer
26.5 μL	H₂O	×17	450.5 μL	H₂O
2.5 μL	T4 ligase	×17	42.5 μL	T4 ligase

The instructor will aliquot 34 μL of this ligation mix to each student. The instructor should have excess cocktail left over. Each student will add 16 μL of their digested DNA from their green PCR tube to their yellow ligation tube.

To set up the iPCR assay for 16 students, you will need:

- 1 orange eppie tube [1.5 mL] (multiplex cocktail)
- 16 orange PCR tubes [0.2 mL] (multiplex)
- 1 tube of Amplitaq Gold 360 2X MasterMix
- 1 tube of iCommon primer
- 1 tube of iPlus primer
- 1 tube of iMinus primer

Make up the one mastermix as follows, making enough cocktail for 17 reactions:

Multiplex each		(# of students + 1)	Multiplex cocktail	
5.5 μL	H₂O	×17	93.5 μL	H₂O
12.5 μL	Amplitaq Gold 360	×17	212.5 μL	Amplitaq Gold 360
1 μL	iCommon	×17	17 μL	iCommon
0.5 μL	iPlus	×17	8.5 μL	iPlus
0.5 μL	iMinus	×17	8.5 μL	iMinus

The instructor will then aliquot 20 μL of the multiplex cocktail into the orange tubes. The instructor should have excess cocktail left over. Each student will then add 5 μL of their ligated DNA from their yellow tube into their orange PCR tube.

The instructor will program the thermal cycler as follows:

- 1 cycle of
  - ○ 98°C   2 min

- 30 cycles of
  - ○ 95℃ 30 s
  - ○ 55℃ 1 min
  - ○ 72℃ 3.5 min
- 1 cycle of
  - ○ 72℃ 7 min
  - ○ 4℃ until collected

Tubes should be put in the PCR machine once the initial denaturation temperature has been reached (98℃).

After PCR amplification, students will add 5 μL 5× BJ to each of their reactions to be used for gel electrophoresis.

Agarose Gel Electrophoresis

The instructor should make up roughly 1 L of 1× TAE buffer per two gels. The TAE buffer suggested for purchase here is 50× concentrated, so for every 1 L, the instructor should mix 20 mL 50× buffer with 980 mL ddH2O, for every 2 L, 40 mL 50× buffer with 1960 mL ddH2O, etc.

The instructor should make up 50 mL of agarose per gel: 50 mL of 1× TAE buffer with 1 g of agarose, once cool, add 35 μL of Sybr Safe, stir, and pour. Be sure to keep the gels away from light until they are to be used. The prepoured gels may be stored in buffer, in a sealed container, protected from light, for up to 1 week.

Instructors may choose for students to pair up, or work in small groups, to load their gels, so the number of gels necessary per class is at the instructor's discretion. Each student will need one well as this is a multiplex reaction.

Students will need 5 μL of 5× BJ for each lane (so 10 μL each, in total). Students may also want to practice loading their gel in spare lanes with 20 μL 1× BJ.

Teaching Laboratory Exercise: Common Pitfalls, Tips, and Tricks

General

The inversion polymorphism that is the focus of this lab has no known phenotypic effect and is of no known medical significance. Though it is incredibly unlikely, it is always possible that students may find out something about their own DNA of which they were previously unaware. If minors will be involved in this laboratory exercise, parental consent should be obtained. College faculty members should check with their Institutional Review Board (IRB) to determine whether IRB approval is required. It is important to make certain that students (and if appropriate—parents) understand any human genetics laboratory exercise and understand that their participation is voluntary. We also strongly discourage attempting to construct pedigrees for students and their families so as to avoid any potential question of parentage.

Mint, coffee, and anything with oils will prevent the PCR reaction from working—it is of vital importance that students rinse their mouths very well before proceeding with the DNA extraction protocol. Students should also avoid coffee and mint during the day of the DNA extraction protocol.

Pipetting a cocktail of mastermix, primers, and water is very important; by making up a cocktail, the instructor can be sure that they have pipetted each reagent into each reaction equally and are not forced to pipette 1 μL of primer into every single PCR reaction. In addition, it is very important that the instructor does not try to make up a cocktail for too many reactions at once, as this decreases the efficiency of each reaction. I suggest making a cocktail for only one section of students at a time (fewer than 20 students). I have made a setup sheet which shows examples of a cocktail for each step.

The setup sheet involves color-coding to limit the likelihood of mistakes. If your institution does not have the same colors or enough colors, I highly suggest that you find a different way to differentiate between tubes.

Contamination will ruin your experiment—make sure that students know to change tips between every single pipetting step.

DNA extractions and PCR are very temperature-sensitive. When possible, please keep tubes and reagents on ice. Please hold tubes in an area where the reagents are not so as not to warm up the enzyme, the DNA, or the primers. If possible, keep tubes on ice between every DNA extraction step (except for the high-temperature steps). For later DNA extraction steps, the ice helps keep things pelleted, so the more time the samples spend on ice the better the pellet will be. All primers should be rehydrated with the appropriate amount of TE buffer provided in the DNA extraction kit (Epicentre cat# MTE0970)

DNA Extraction

You will need to add 1 μL of Proteinase K (PK) solution to each student's purple eppie tube. I suggest adding the PK after the students have twirled their swabs in the buffer. Be sure to change tips between every tube and to check that you've gotten PK into the tube (by checking the tip before and after adding PK to each purple tube).

You will need to add 1 μL of RNase A solution to each student's purple eppie tube. I suggest adding the RNase after the tubes have been at 37℃ for 5 min. Be sure to change tips between every tube and to check that you've gotten RNase into the tube (by checking the tip before and after adding RNase to each purple tube).

LongAmp PCR Assay

The most common mistake that students make is mixing up the reactions on their gels by pipetting their plus reaction into their minus lane and their minus reaction into their plus lane. Students must be very careful to pipette the reactions in the correct order, or to at least take very clear notes about which reaction is in which lane. I recommend that all students load their plus reaction in their left lane, and their minus reaction in their right lane.

This is a very fickle protocol. If some reactions are not robust enough, the instructor may re-run the PCR reactions with either (a) a longer extension time, or (b) more cycles. The current protocol is the shortest run time that produces generally reliable results, which is why it was chosen, but if you are running the PCR overnight anyway, adding 2 more hours to the run is preferable.

In many individuals, a band appears in the plus lane that is significantly smaller than the plus amplicon. This is a secondary product that occurs due to the repetitive nature of this region and is NOT indicative of a plus genotype. The band MUST appear in the ~12 kb region to represent a plus X chromosome.

iPCR Assay

The iPCR assay involves a self-ligation step. In order to enhance self-ligation (and not ligation to other fragments), it is important to have a low concentration of DNA in the ligation step.

Agarose Gel Electrophoresis

Loading an agarose gel can be difficult and anxiety-inducing for some students. I recommend providing the students with 1× blue juice that they can practice with. You can either give them extra lanes in their gels or provide a practice gel at the front bench. Students can practice pipetting into these gels until they feel comfortable pipetting their own samples.

SYBRSafe is light-sensitive—if you have trouble visualizing bands or even ladder, turn out the lights and close the blinds. Author C. S. Kirby provides the first-person suggestions throughout the laboratory exercise.

From Sex Linkage to Genomics—A Genetic Mother Lode

Laboratory Exercise Discussion Questions

Students will endeavor to learn core molecular biology techniques such as iPCR, long PCR, restriction enzyme digest, ligation, and gel electrophoresis. Students will also learn how to analyze a gel, interpret presence-absence assays, do HWE calculations, relate classroom data to concepts in the real world, and how to ask good questions about gaps in scientific knowledge.

1. Why are long, nearly identical inverted repeats (IRs) found disproportionately on the X and Y chromosomes (see Warburton et al. [4])?
   a) The X and Y chromosomes have no homologs with which to pair over most of their lengths leading the IRs to pair with themselves and undergo gene conversion to maintain near identity.
2. Many, but not all, of the long IRs, whether X-linked, Y-linked, or autosomal, contain genes which are expressed only or primarily in testes. Discuss this observation.

a) For example, is there some advantage to having multiple copies of these genes (which are involved in spermatogenesis and implicated in cancer) in males?
3. Why do we think non-allelic homologous recombination (NAHR) on the X-chromosome occurs more frequently in males than in females?
   a) The "sex & the single X" - again, no homolog in males for the X-chromosome so the IRs pair with themselves leading to Holliday junctions and crossing over.
4. Why do we question the apparent lack of polymorphism for the filamin-emerin inversion in other eutherian mammals (Caceres [2])?
   a) The inverted repeats are nearly identical in almost every eutherian mammal studied. The near identity is suggestive of heteroduplex formation and gene conversion. Holliday junctions should be forming in conjunction with the heteroduplex and the resolution of these junctions should lead in some cases to crossing over and inversion of filamin and emerin.
5. In the text of this article, two models are presented for the origin of the emerin deletion/partial filamin duplication in the Emery-Dreifuss muscular dystrophy patient—a double crossover in a plus homozygote or a single crossover in a plus//minus heterozygote. Argue the relative merits of these two models including the role of improper pairing in the inverted region in producing the unequal crossover.
   a) Small model:
      i. pros:
         1. females of European ancestry are most commonly +//+ homozygotes.
         2. mispairing of the LIRs could lead to mispairing in the emerin-filamin region and result in an unequal crossover.
      ii. cons:
         1. chromosome interference makes a double crossover in this tiny space very unlikely.
   b) Our model:
   i. pros:
         1. +//− heterozygotes are less common but not uncommon in individuals of European ancestry.
         2. inversion heterozygosity could lead to mispairing in the emerin-filamin region and result in an unequal crossover.
         3. no second crossover is needed, so chromosome interference is not a factor.
6. Calculate arrangement frequencies and check for Hardy-Weinberg using the following data set:

	+//+	+//−	−//−	Total
Females	42	36	22	100
	+/y	−	−/y	−
Males	60	−	40	100

If the females deviate significantly from Hardy-Weinberg equilibrium, what are some possible explanations for the deviation?

Allele frequencies = (2 # homozygotes + # heterozygotes + # hemizygotes)/(2 # females + # males)

a. Plus: 0.6 Minus: 0.4
b. HWE test: if plus = 0.6 and minus = 0.4

Then, expected $0.6 \times 0.6 \times 100 = 36$ plus, $0.6 \times 0.4 \times 2 \times 100 = 48$ het, $0.4 \times 0.4 \times 100 = 16$ minus

$\chi^2 = ((42 - 36)^2/36) + ((36 - 48)^2/48) + ((22 - 16)^2/16) = 1.0 + 3.0 + 2.25 = 6.25$ with 1 degree of freedom yielding significant deviation at 0.05 level.

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