Chapter 24

Capillary Electrophoresis for the Detection of Fragile X Expanded Alleles

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Abstract

Capillary electrophoresis is an analytical technique that separates ions based on their electrophoresis mobility with the use of an applied voltage. Capillary electrophoresis is used most predominantly in nuclear acid fragment analysis as well as DNA sequencing because it gives faster results and provides high resolution separation. Here we describe an application using capillary electrophoreses for screening the Fragile X expanded alleles to demonstrate the application.

Key words: Capillary electrophoresis, Fluorescent detection, Fragile X

1. Introduction

Capillary electrophoresis (CE) was introduced almost 30 years ago by Jorgensen et al. as a new and automated alternative to slab gel electrophoresis (1). As early as 1989, Bob Brownlee and coworkers introduced the first commercial instrument with on column UV/VIS detection, automatic injection, and computerized data analysis for rapid, high resolution CE separation (2). Since that time, others followed the lead in manufacturing commercial CE units, including several companies developing capillary-based DNA sequencers. The CE is the most successful development of an analytical tool for high-throughput, cost-effective, and reliable DNA analysis. The advantage of CE, when compared to other liquid-phrase separation techniques, is the analysis speed combined with the extraordinarily high separation efficiency.

In this type of electrophoresis, the analysis is resolved in a thin glass (fused silica) capillary that is 30–100 cm in length and has an

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internal diameter of 25–100 μm. The capillary is covered with a polyimide coating for protection. There is an uncoated window where light is shone on the fragment as they pass the detector. The capillary has a negative charge along its walls generated by the dissociation of hydroxyl ions from the molecules of silicone. This establishes an electro-osmotic flow when current is introduced along the length of the capillary. Under the force of the current, small and negatively charged molecules migrate faster than large and positively charged molecules. Capillary electrophoresis was originally applied to molecules in solution. Separation was based on their size and charge (charge/mass ratio). Optimal separation requires the use of the proper buffer to ensure that the solute is charged. Negatively charged molecules are completely ionized at high pH, whereas positively charged solute is completely prorogated in low pH buffers.

Nucleic acids do not separate well in solution. As the size or length of a nucleic acid increases, its migration is retarded, effectively confounding the charge/mass resolution. Introduction of a polymer inside the capillary restores resolution by retarding migration according to size more than charge. It is important that nucleic acid be completely denatured (single-stranded) (3, 4).

In narrow capillaries with high electric resistance and high capacity to dissipate Joule heating, electric field strengths as high as 1 kV/cm can be used. Consequently, the analysis time is typically one order of magnitude shorter than that found in conventional slab gel electrophoresis. Another remarkable feature of CE is that the operational conditions can be kept constant, defined, and reproducible for weeks by filling the capillary with fresh electrolytes prior to each analysis. The analytical potential of CE has been proven by the massive implementation in the Human Genome Project (HGP) in which about three billion base pairs (bp) of human DNA were sequenced de novo utilizing capillary sequencers (3–5).

Capillary electrophoresis has broad applications in molecular biology in nucleic acid detection and sizing (6, 7). Fluorescently labeled primers amplify segments of DNA by polymerase chain reaction (PCR). The fluorescently tagged products are injected into a capillary for separation. The single base resolution of polymers used in the capillary allow for DNA or cDNA sequencing applications. The high resolution also allows for separation of differently sized products. An example is short-tandem repeats segments of DNA such as is seen in Fragile X syndrome (FX).

FX is the most common inherited form of mental retardation and related intellectual and developmental disabilities. Symptoms include mental retardation, long face, and prominent ears with males being more severely affected than females (8). The cause is a triplet repeat expansion (CGG)n in the 5′ untranslated region of the *FMR1* gene. If this repeat is expanded beyond 200 repeats, the

region becomes hypermethylated which silences expression of the gene.

Carriers of a smaller, unmethylated, yet unstable allele are said to have premutations. The repeat size is approximately 56–200 CGG repeats. Females who are premutation carriers have a risk of the allele expanding in the oocytes into the full mutation in their offspring (9). The risk for expansion increases with increasing sizes of premutations, with nearly 100% expansions to full mutations from alleles with 100 or more repeats (10).

Premutation carriers may also be at risk for adult onset conditions. Females are at risk for premature ovarian insufficiency (POI), while males may develop FX-related ataxias and tremors (FXTAS) (11, 12).

Testing traditionally has included two methods, PCR to accurately size normal and smaller premutation alleles and Southern analysis to detect larger premutations and full mutations and to assess methylation status. Southern analysis is labor intensive which makes it unsuitable for high-throughput and quick turn-around time. Recently described assays called triplet-primed repeat PCR combined with capillary electrophoresis simplifies detection of FX expanded alleles (13–15). In triplet-primed repeat PCR, one primer flanks the CGG repeat region, while the other can bind within the CGG repeat (termed chimeric primer). PCR products are amplified from each of the CGG repeats. When electrophoresed in agarose, the multiple products appear as a smear (16). When combined with the high resolution of capillary electrophoresis, the smear becomes a characteristic stutter pattern. This stutter will extend as far as the highest CGG repeat allele. If it extends into the premutation range (>55 repeats), the sample is considered positive for an expanded allele and reflexed to sizing PCR and Southern analysis.

2. Materials

Although several methods have been described and alternative CE equipment is available, we include reagents and instruments that we routinely use, namely CE instruments from Applied Bioscience, Fragile X primers from Celera (distributed by Abbott), and analysis software from SoftGenetics. This following procedure however, can be adapted to other methods (14, 15), and instruments.

2.1. Reagents

1. High GC PCR buffer: Mix of dATP, dCTP, dGTP, dTTP, betaine, and 2-pyrrolidinone.
2. Fragile X primers.
3. Hot start *Taq* polymerase.

4. Rhodamine-X labeled ROX 1000 Size Standard or MapMarker 1000 (50–1,000 bp—see Note 1).
5. 10× TBE buffer.
6. HiDi Formamide.

2.2. Disposables

1. 1.5 mL microfuge tubes.
2. 1–5/5–50/50–200/200–1,000 µL barrier pipet tips.
3. 36 cm capillary array.
4. Plastic plate sealer sheets.
5. MicroAmp Optical 96-well reaction plate.

2.3. Equipment

1. Thermocycler.
2. Micropipets, 1–1,000 µL.
3. High speed microfuge for 1.5 mL tubes.
4. Microplate centrifuge.
5. Capillary electrophoretic instrument such as ABI Prism® 3100, 3130 Genetic Analyzer with 36 cm capillary array.
6. Software for genetic analysis such as GeneMarker software.

3. Methods

In high-throughput FX screening, a single PCR using a "chimeric" primer (Fig. 1) within the *FMR1* gene and size separation by CE determines the presence or absence of an expanded *FMR1* allele. By evaluating stutter peak patterns, premutations, full mutations, and mosaics are discernable as an expanded allele while alleles with repeats in the normal range are clearly distinguished as not expanded. Individuals with expanded alleles should be evaluated with sizing PCR and Southern blot analysis to determine precise allele sizing and methylation pattern.

3.1. DNA Isolation

DNA can be extracted with any method. DNA concentration for this method is approximately 10–25 ng/µL.

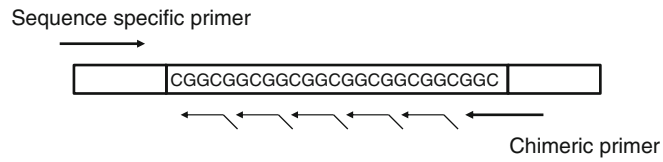

Fig. 1. Diagram of FX screening concept. The sequence-specific primer amplifies from one direction of the FMR1 gene. The chimeric primer extends into the CGG repeat region and therefore will bind and amplify from every repeat.

Table 1
PCR mix preparation for FX screen

Reagent	Volume, μL for 1 reaction (×1)
DNase/RNase-free water	1.7
High GC PCR buffer	13.0
Fragile X primers-II	0.8
TR PCR enzyme mix	1.5
Total	17.0 μL

3.2. PCR Setup

1. In a designated area (clean room), thaw all reagents and vortex thoroughly.
2. Prepare the PCR master mix according to Table 1, multiplying each reagent volume by the number of reactions required (see Note 2). Place master mix into a 0.6-mL microcentrifuge tube.
3. For quality control purposes, the following controls are added to each run:
   (a) Normal allele (normal female with one allele at ~45 repeats).
   (b) Expanded allele (premutation female).
   (c) Reagent negative (no template added).

3.3. PCR Reaction Setup

1. Mix the water, buffer and primers to the tube first.
2. Add the enzyme and mix by flicking the tube two times, then spin it down.
3. Aliquot 17.0 μL of master mix into each reaction tube.
4. Seal the plate with a plastic sheet.
5. Briefly vortex and spin down each DNA sample and control.
6. Following the tray map, add 3.0 μL of DNA into the respective reaction tube (see Note 3).
7. Mix by pipetting three to five times.
8. Re-cap the plate with the plastic sheet.
9. Move the plate to a designated area (Amplification/Detection Room).
10. Start the thermocycler program to warm up lid and block.
11. Spin down the tray briefly.
12. Place plate on the thermocycler when the block reaches 95°C. Run the amplification program as described in Table 2. PCR takes about 2 h.

Table 2
PCR thermocycler program

Step	Temperature, °C	Time	Cycle
Denature	95.0	5 min	1
Denature	98.5	30 s	
Annealing	53.0	30 s	50
Extension	75.0	1 min	
Hold	4.0	Infinitely	Hold

Table 3
Sample loading master mix

Reagent	Volume, μL for 1 reaction (×1)	Final concentration
HiDi formamide	20.0	NA
Size standard	2.0	1×
Total	22.0	

3.4. Sample Preparation for Loading Onto CE

1. Thaw HiDi formamide.
2. Prepare a HiDi formamide/MapMarker 1000 size standard master mix according to Table 3 (see Note 4).
3. Vortex briefly and spin down.
4. Aliquot 22.0 μL of the formamide/size standard mix into each well of a barcoded MicroAmp Optical 96-well reaction plate.
5. Add 2.0 μL of PCR product to the respective reaction wells.
6. Secure a clean and dry septa strip on the sample plate.
7. Heat to denature the loading mix for 2 min at 95°C.
8. Place on cold block until ready to load onto the ABI instrument.
9. Place the plate onto the ABI 3,100×1 or 3,130×l Genetic Analyzer.

3.5. Instrument Setup

1. Follow the manufacturer's instructions for CE instrument setup.
2. Check if there is enough polymer left in the syringes and refill polymer if necessary (see Note 5).
3. Change the buffer and water in the reservoirs (see Note 6).

Table 4
Run module settings

Name	Value	Range
Oven_Temperature	60	18–65°C
Poly_Fill_Vol	6,500	6,500–38,000 steps
Current_Stability	5.0	0–2,000 uA
PreRun_Voltage	15.0	0–15 kV
Pre_Run_Time	180	1–1,000 s
Injection_Voltage	15.0	1–15 kV
Injection_Time	8	1–600 s
Voltage_Number_Of_Steps	20	1–100 nk
Voltage_Step_Interval	30	1–60 s
Data_Delay_Time	240	1–3,600 s
Run_Voltage	15.0	0–15 kV
Run_Time	2,100	300–14,000 s

4. Start with a new plate and enter the information such as sample type, size standard, the assay to run, analysis method, results setup, and instrument protocol.
5. Link the correct run name to the correct plate name.
6. Samples are injected under conditions see Table 4 for the run module.

3.6. Data Analysis with Gene Marker software

1. Open the GeneMarker software.
2. Click on *Open Data*, then *Add* to select the files for analysis, and then load the files.
3. Enter the CE parameters used for Fragile X screening (see Note 7).
4. Select the following parameters:
   (a) Raw data analysis:
      - Auto range (frame).
      - Smooth.
      - Peak saturation.
      - Baseline subtraction.
      - Pull-up correction.
      - Spike removal.
   (b) Size call:
      - Local southern.

(c) Allele call:

- Auto range (bps), start: 35, end: 1,000.
- Intensity >100; Percentage >5 Max.
- Local region % >25 Local Max.
- Max Call Intensity: 30,000.
- Stutter peak filter: left = 90; right = 40.

5. Select the following parameters:
   (a) Allele Ladder: none.
   (b) Allele Evaluation. Peak Score: Reject <1 Check 7 < Pass.
6. Set axis range:
   (a) Adjust the *X*-axis from 35 to 1,000.
7. To see enlarged Fig. 2 insets, change the *X* and *Y*-axes by justify the *Axis Range*.
   (a) Adjust the *X*-axis from approximately 265 to 365.
   (b) Adjust the *Y*-axis from approximately –250 to 2,000 (or the highest in the region).

4. Result Interpretation

Figure 2 shows typical results of samples with a normal (not expanded) allele and an expanded allele. A no-template control is also shown. The range of negative of CGG repeat is below 45 repeats. Repeat sizes from 45 to 55 are considered intermediate. Repeat sizes in this range have not been reported to expand to a full mutation in one generation. However, they may be unstable and expand into the premutation range.

1. Negative: an allele in the normal repeat range generally has a distinct drop off of fluorescence and a flat line to the end of the electrophorogram. Stuttering beyond 300 bp is not observed.
2. Positive: an allele in the expanded range (premutation and full mutation) will stutter beyond the 300 bp range.

An intermediate allele is not considered expanded, but if identifying carriers of intermediate alleles is desired; the cut-off can be lowered to 44 repeats.

5. Notes

1. MapMarker 1000 internal size standard is designed to size DNA fragments between 50 and 1,000 bp using 23 fragments

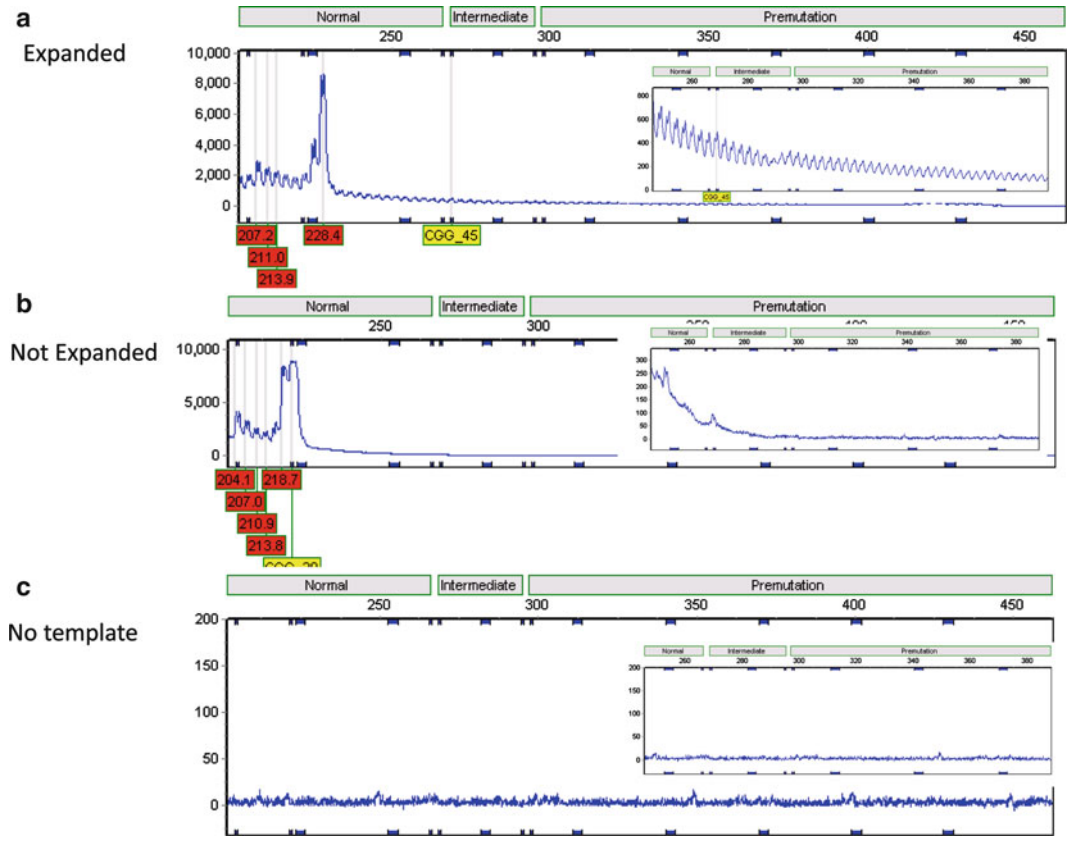

Fig. 2. Electropherograms showing the characteristic patterns for (**a**) an expanded allele, (**b**) an allele that is not expanded, and (**c**) a no-template control. The insets show an enlargement of the region into the premutation range (>55 CGG repeats).

of: 50, 75, 100, 125, 150, 200, 250, 300, 350, 400, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, and 1,000 bp (Applied Bio).

2. Calculate the number of reactions needed by adding each sample, control and one to two dead volumes (5 sample + 3 controls + 1 dead volume = 9 reactions).
3. For the reagent negative, do not add any additional material.
4. Multiply each reagent volume by the number of reactions required plus one or two dead volumes.
5. Polymer must be less than 7 days old. The ABI 3100 or 3130 instrument, for example, is loaded with pop-7.
6. This must be done daily at a minimum or before each run.
7. These parameters are Fragile X screening panel, sized standard used for CE and the color of the fluorescent dyes and specimen type.

Common scenarios and possible troubleshooting resolutions.

5.1. Low DNA Concentration

It is the most common cause failure of the assay. It could be due to the DNA extraction failure or the minimum amount of the DNA obtained from the specimen, for example the fetal specimen. The resolution is check the DNA concentration by spectrophotometer. Once it is confirmed, repeat DNA extraction if required. If the DNA concentration is low, run sample in duplicate inoculating one reaction with 2 μL of genomic DNA and the duplicate with 4 μL.

5.2. Spike Obscures Results

Air bubbles or polymer particles in the injection can create spikes. The troubleshooting is to adjust the Υ-axis and reprint.

5.3. PCR Failure

Incomplete mixing of the viscous 50% glycerol enzyme solutions with dilution buffers are a major source of errors and can lead to PCR failures. The PCR needs to be repeated.

Or poor pipetting. It needs to always check the volume of liquid in the pipet tip before dispensing and pipet directly into a reaction. Repeat injection, including a longer injection if failures are in the larger fragments.

5.4. No Bands Are Visible When Analysis Is Completed

Size standard was not added or mobility affected by contaminant although it can be caused by PCR failure, extraction failure, and too much DNA added to reaction. Check the size standard pattern and repeat injection if it failed. Check PCR primer-dimer peaks (~50 bp). Check DNA concentration and repeat from PCR or extraction.

5.5. Bands Are Visible, But Signals Are Weak

It can be caused by DNA impurities. Check A260/A280 and A230/A260 ratios of the DNA and dilute samples to lower concentration if ratio is out of purity range. Otherwise, the DNA needs to be re-extracted for the specimen

5.6. Assay Variation

For the capillary electrophoresis, the injection time may vary within a few seconds. It is affected by the sensitivity of the instrument, robustness of the assay, and quantity of salts, or other low molecular weight ionized products in the reaction(s).

6. Method Limitations

Rare mutations in the *FMR1* gene, unrelated to trinucleotide expansion, may not be detected. Specificity may be compromised by rare primer site mutations.

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