A Simple Screening Assay for C9orf72 ALS Repeat Expansions

sage science

Application Note: SageELF

A fast and inexpensive qPCR-based method for screening repeat expansions

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Introduction

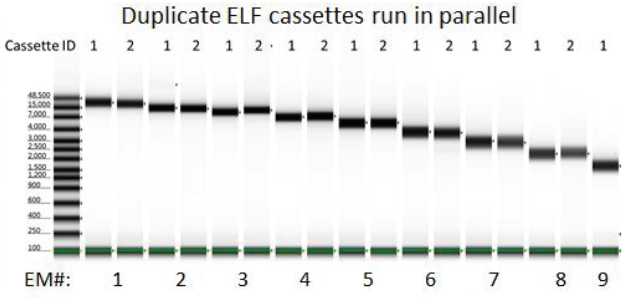
The hexanucleotide repeat expansion (RE) in C9orf72 is the most common genetic biomarker of familial ALS-FTLD. In unaffected populations, the repeat region is short (2- 24 repeats of the G4C2 hexanucleotide repeat unit) and can be identified with routine DNA sequencing. In contrast, ALS-FTLD patients have REs with hundreds to thousands of repeats. In ALS, longer REs can correlate with the age of onset, the severity of clinical symptoms, or the mechanism of disease. The heterogeneity of clinical phenotypes in ALS also suggests the possibility of disparate responses to therapeutics, so accurate methods for RE characterization could have great benefit for ALS research, diagnostics, and therapy management.

We present a simple RE typing procedure that uses the SageELF electrophoretic platform to size-fractionate and collect DNA. Briefly, genomic DNA is digested with restriction enzymes that cleave in single copy regions flanking the repeat. The digest is separated into 12 consecutive size fractions on an automated preparative electrophoresis system. The size fractions containing the RE region are identified by qPCR, using a single-copy amplification target located adjacent to the repeat. The length of the repeat expansion can be determined directly from the size fraction in which it is located. All assay steps (digestion, fractionation, and qPCR) can be carried out in a day.

The SageELF

The SageELF system performs preparative DNA size selection and elution in an automated fashion. For a given set of electrophoresis settings, the eluted fragment size can be determined from elution module position.

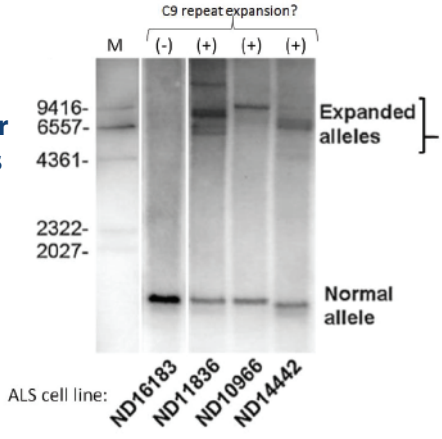
SageELF system and gel cassette schematic



Agarose gel analysis of DNA size fractions in elution modules (EM).

Current Methods for C9orf72 RE Analysis

These existing methods are either labor-intensive and lengthy (in the case of Southern blotting), or require expensive specialized equipment (in the case of RP-PCR).

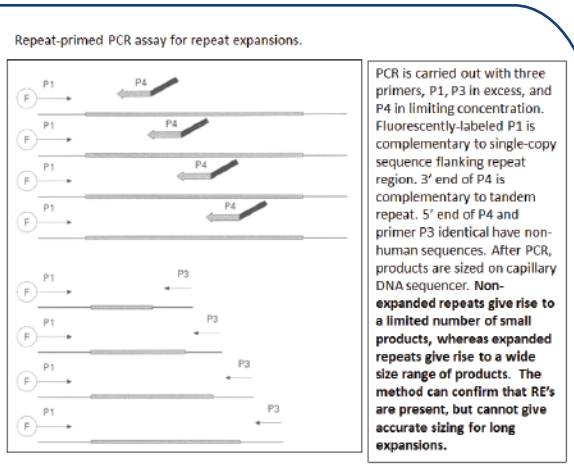


Modified from Liu, et al., 2014, Acta Neuropathol. 128(4):525-541; Fig. 3C.

Southern Blotting

Genomic DNA was restricted with XbaI, Southern blotted, and hybridized to non-repetitive probes for 2.4kb fragment carrying the G_4C_2 repeat.

RE fragments 4.3kb to 9.6kb contain expansions of ~315-~1170 hexamer repeats (GGGGCC).



PCR is carried out with three primers, P1, P3 in excess, and P4 in limiting concentration. Fluorescently-labeled P1 is complementary to single-copy sequence flanking repeat region. 3′ end of P4 is complementary to tandem repeat. 5′ end of P4 and primer P3 identical have non-human sequences. After PCR, products are sized on capillary DNA sequencer. **Non-expanded repeats give rise to a limited number of small products, whereas expanded repeats give rise to a wide size range of products. The method can confirm that RE's are present, but cannot give accurate sizing for long expansions.**

Adapted from Warner et al., 1996, J. Med. Genet. 33:1022-1026.

RP-PCR

SageELF RE Screen Workflow

The schematic below shows an overview of the workflow used. The entire process can be completed in approx. 8 hours. DNA size fraction collection with the SageELF requires 2-3 hours for these size ranges, but may require longer for larger RE screens.

Blood or tissue sample

⬇

Isolate HMW DNA

⬇

Restriction digest -- cut single-copy sequences on both sides of repeat region.

⬇ 1.5 hrs

Electrophoretic size selection (separate unexpanded and expanded repeat fragments). Isolate of DNA from 12 sequential sections of gel lane.

⬇ 2-3 hrs

qPCR using single copy target on fragment containing repeat region (qPCR target lies on one side of repeat – not across repeat)

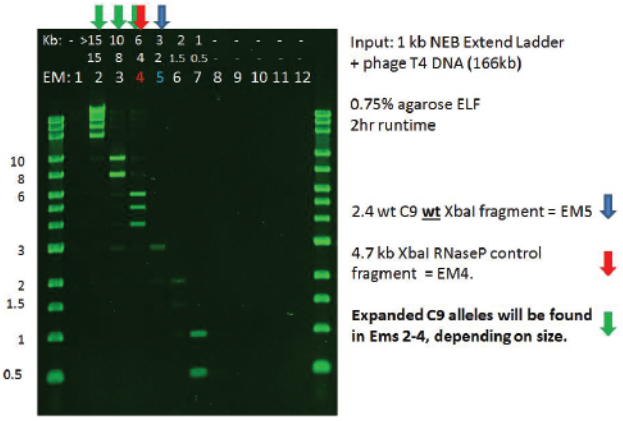
⬇ 2.5 hrs

Determine presence/size of repeat expansion from ***position*** of qPCR signal in elution fractions.

(Confirm positive tests by repeat-primed PCR or sequencing.)

SageELF Electrophoresis Conditions

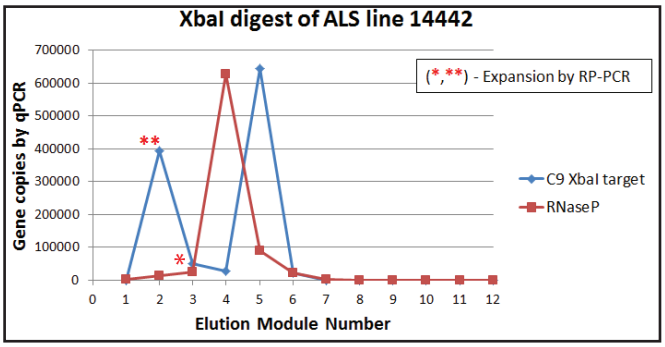
The wt C9 XbaI fragment is 2.4kb in size, when 2-3 G4C2 repeats are present. Southern blot analyses suggest that most ALS patient have expansions ranging in hundreds to thousands of repeats. XbaI fragments carrying such expansions would be found in elution modules 2-4.

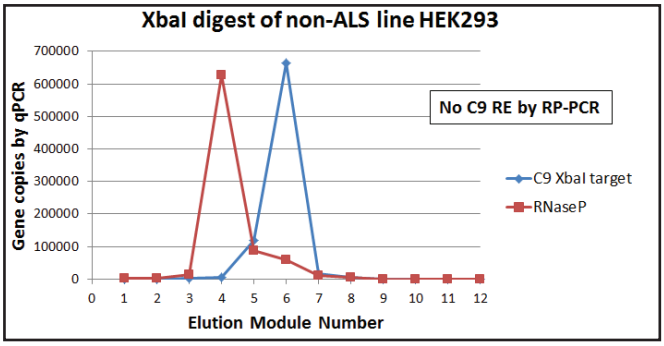


Agarose gel analysis of collected DNA ladder fragments

Results

Results indicate the presence of target in collected size fractions of the XbaI digest. These are indicative of the C9orf72 ALS RE in the expected elution modules. The postive indications were confirmed by RP-PCR.





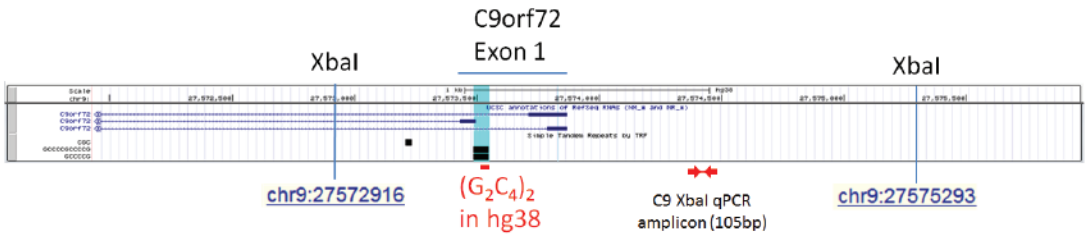
qPCR results of RE screenng assay

Discussion

Our assay combines the benefits of Southern blotting for RE sizing, with the sensitivity of PCR, without the need to amplify through the repetitive 100% GC-rich repeat region. Since the electrophoretic resolution can be tailored to different size ranges by changing gel concentration, voltage, and run time, our assay may also be useful for characterizing repeat lengths in other RE diseases. This could include diseases with smaller repeat expansions such as Huntington's Disease, Fragile X syndrome, Friedrich's ataxia, etc.

Genomic Map of C9orf72 Repeat Region

XbaI was used to excise the first exon region of C9orf72. In the hg38 reference, this XbaI fragment is 2377 bp, and there are two G2C4 repeats. The 105 bp qPCR amplicon used for detection of the C9 XbaI fragment is located ~1kb to the right of the G2C4 repeats.



XbaI cut sites, RE region, and 160bp qPCR target