A Simple Screening Assay for C9orf72 ALS Repeat Expansions

Chris Boles[1], James Gregory[2], Jun Zhou[1], Kristy Kang[2], and Hemali Phatnani[2]

[1]Sage Science, Inc., Beverly, MA;

[2]Center for Genomics of Neurodegenerative Disease, New York Genome Center, New York, NY

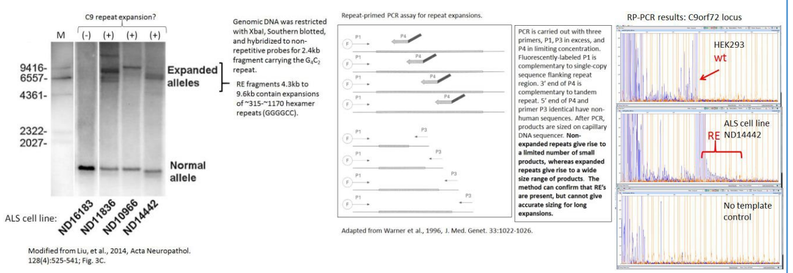
Abstract

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 G_4C_2 hexanucleotide repeat unit) and can be identified with routine sequencing. In contrast, ALS-FTLD patients have RE's with 100's to 1000's 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 a novel electrophoretic method. Briefly, genomic DNA is digested with restriction enzymes or customized Cas9 nucleases 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.

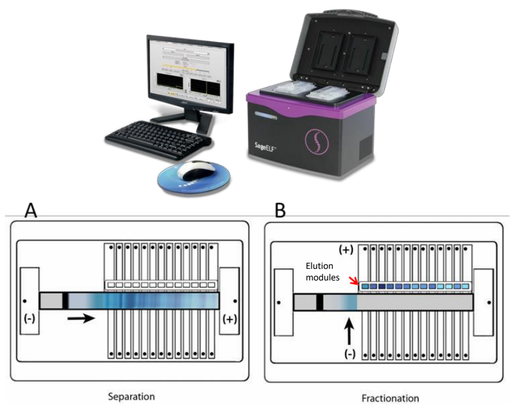
Our assay combines the benefits of Southern blotting for RE sizing, with the sensitivity of PCR, without the need to amplify through the repetitive 100% GC-rich repeat region. Since the electrophoretic resolution can be tailored to different size ranges by changing gel concentration, voltage, and run time, our assay may also be useful for characterizing repeat lengths in other RE diseases.

Conventional methods for C9orf72 repeat expansion

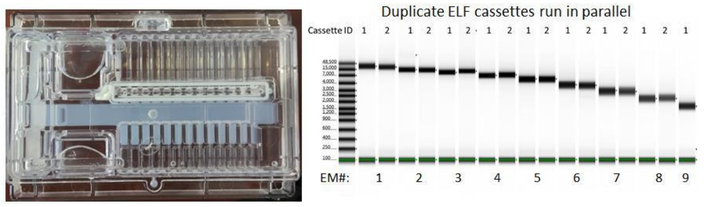


These existing methods are either labor-intensive and lengthy (in the case of Southern blotting), or require expensive specialized equipment (in the case of RP-PCR).

A new screening assay based on SageELF preparative platform



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. An example of SageELF DNA fractionation is shown below.



Overview of SageELF workflow for RE screening

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)

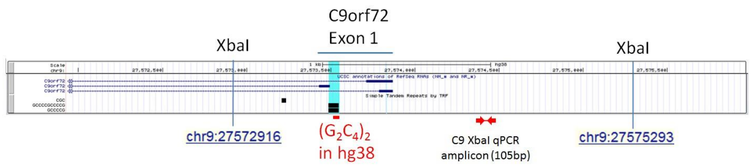
↓ 2.5 hrs

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

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

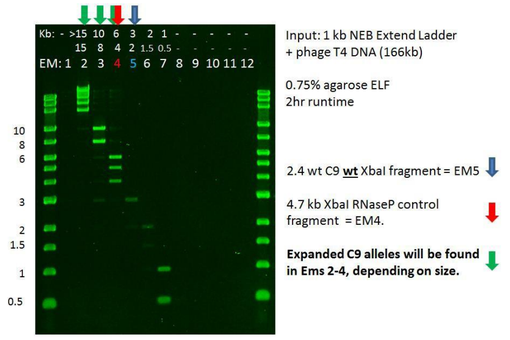
Genomic map of C9orf72 repeat region

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 G_2C_4 repeats. The 105 bp qPCR amplicon used for detection of the C9 XbaI fragment is located ~1kb to the right of the G_2C_4 repeats.



Electrophoresis conditions used for C9orf72 testing

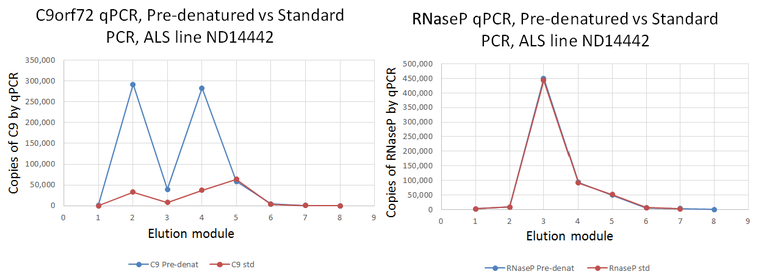
The wt C9 XbaI fragment is 2.4kb in size, when 2-3 G_4C_2 repeats are present. Southern blot analyses suggest that most ALS patient have expansions ranging in 100's to 1000's of repeats. XbaI fragments carrying such expansions would be found in EMs 2-4.



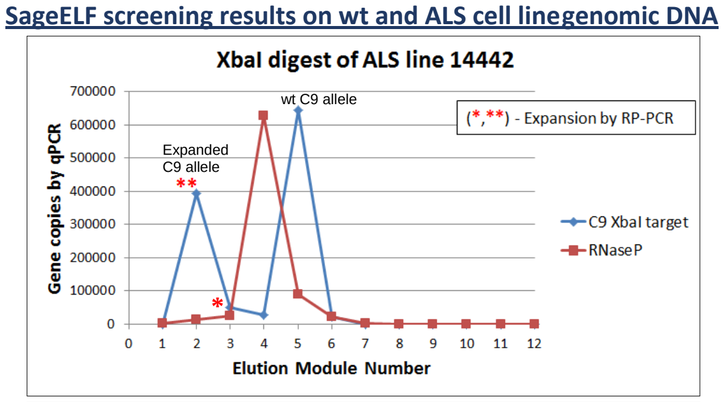
Process controls and assay refinements

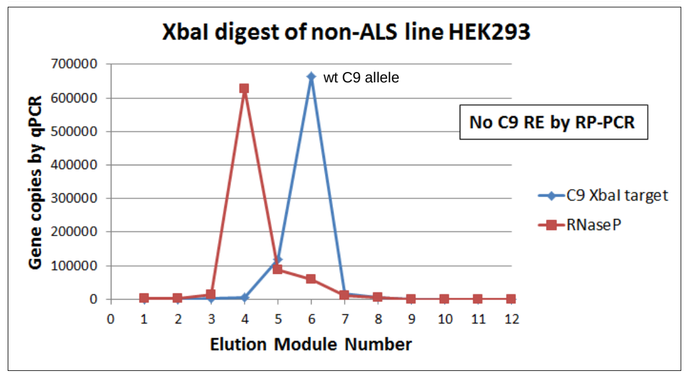
Since incomplete restriction digestion will produce wt C9 XbaI fragments that cannot be distinguished from true repeat expansions on the basis of size alone, control digestions were performed in which a small portion of each restriction reaction was mixed with a small excess of phage M13 DNA to indirectly monitor extent of reaction. The M13 test digestion was analyzed on agarose minigels or Agilent Bioanalyzer before proceeding to ELF loading. We also analyze a different single copy gene (RNaseP) in parallel with C9, to enable detection of partial digestion.

We found that the C9orf72 2.4kb XbaI fragment (wt or expanded) would not give accurate, reproducible qPCR signals, unless the digest was vigorously denatured by boiling for 10 minutes (in TE) before adding the DNA to the qPCR reaction. The vigorous denaturation had no effect on the control gene amplification (RNaseP).

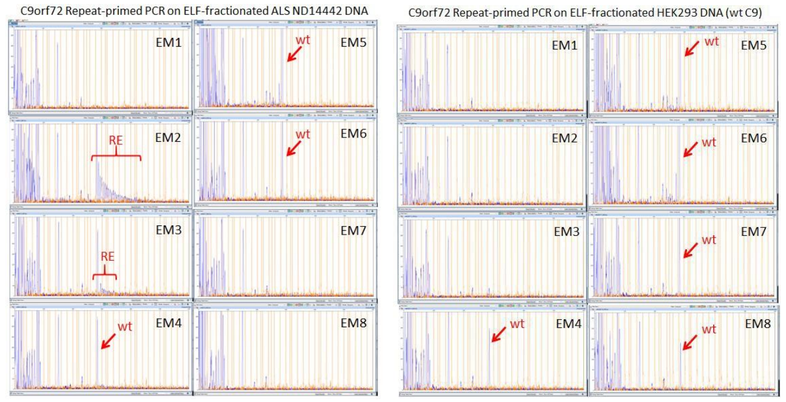


SageELF screening results on wt and ALS cell line genomic DNA





Confirmation of expansion status by repeat-primed PCR

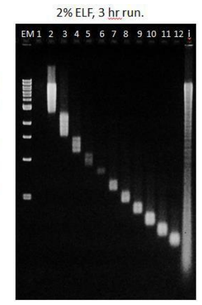


Applications to other repeat expansion diseases

By changing gel concentrations and other electrophoresis conditions, the SageELF can also resolve smaller DNA fragments, and therefore could be used for other diseases such as Huntington's Disease, Fragile X syndrome, Friedrich's ataxia, etc., which have smaller repeat expansions. An example of an ELF separation on sheared genomic DNA using a 2% agarose gel is shown below.

Size of Eluted Products

Lane	From [bp]	To [bp]
1	No DNA	
2	2,058	14,930
3	1,413	3,831
4	987	2,397
5	834	1,481
6	735	963
7	536	736
8	428	591
9	343	480
10	259	395
11	197	307
12	139	248



Contact information

Technical questions:

Chris Boles – chris.boles@sagescience.com

James Gregory -- jgregory@nygenome.org

Sage sales:

info@sagescience.com

www.sagescience.com www.nygenome.org