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WHITE PAPER

Comparison of Capillary and Agarose Electrophoresis Profiles using the AmplideX® PCR/CE *DMPK* Kit* with Normal and Expanded Genomic DNA Samples

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ABSTRACT

Triplet repeat CTG expansions in the *DMPK* gene cause Myotonic Dystrophy Type 1. Current laboratory workflows require PCR and Southern blot analysis to adjudicate DM1 repeat status. To simplify this workflow, Asuragen developed a single-tube, long-read PCR assay based on AmplideX® technology. This assay accurately sizes ≤200 CTG repeats and flags larger expanded alleles by both presence of a stutter pattern and pile-up peak using capillary electrophoresis (CE). Larger expansions that cannot be sized using CE may be resolved using an agarose gel electrophoresis (AGE) method. As a result, this single PCR technology unifies gene-specific and repeat-primed designs in a streamlined procedure to genotype both normal and expanded alleles. The objective of this study was to compare PCR/AGE results from the AmplideX PCR/CE *DMPK* Kit* with a comparator method using clinical samples, and provide examples of the associated molecular profiles and their interpretation.

INTRODUCTION

Myotonic Dystrophy is the most common adult-onset muscular dystrophy, and the most severe form is type 1 (DM1). The autosomal dominant disease is caused by a pathogenic CTG expansion in the 3' UTR of the myotonic dystrophy protein kinase gene (*DMPK*). In the absence of family history, early DM1 symptoms are typically mild, overlapping with other disorders (e.g. baldness, myalgia, cataracts) which leads to under- or misdiagnosis of patients[1]. With the introduction of molecular testing, disease prevalence could be as high as ~1:2000[2].

In clinical labs, DM1 molecular testing requires both PCR and Southern blot (SB) analysis. Most laboratory-developed PCR tests (LDTs) cannot consistently amplify >100 repeats. They also require multiple reactions for either expansion detection or sizing due to amplification dropouts. Not only do SB assays take approximately one week to perform, but require radio-labeled reagents and are highly variable between operators and runs. Here, we describe a novel PCR amplification technology that overcomes these hurdles and reliably resolves normal and expanded alleles using capillary electrophoresis (CE) for ≤200 repeats and flags >200 in a single reaction. We detail the performance of an optional AGE method developed for size estimation and categorical genotyping for many larger expansions. We then compare the results for samples tested by both SB and PCR/AGE.

AmplideX® PCR/CE *DMPK* Kit*

The AmplideX® PCR/CE *DMPK* Kit* is an *in vitro* nucleic acid amplification kit for the analytical assessment of CTG repeats in the 3' untranslated region of the *DMPK* gene (**Figure 1A**). The kit employs polymerase chain reaction (PCR) on extracted deoxyribonucleic acid (DNA) followed by capillary electrophoresis (**Figure 1C**). The kit generates numerical values for alleles up to and including 200 repeats and a categorical value for alleles >200 repeats to facilitate genotyping.

For size estimation beyond 200 repeats, the kit provides instructions to perform an optional PCR followed by AGE.

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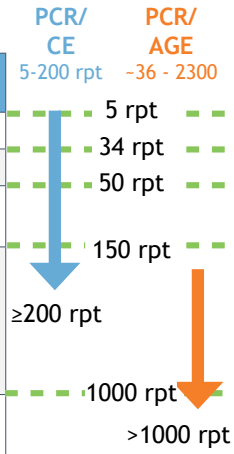
MATERIALS AND METHODS

Samples and Sample QC

A total of 67 residual clinical DNA samples were selected with genotypes independently determined using PCR (5-100 repeats) and SB (up to 3700 repeats) at a reference lab. Samples were selected to provide multiple representatives in each numerical category throughout the dynamic range. Samples covered each of the clinical categories ≥35 CTG repeats (**Table 1**). PCR/CE was performed on all samples and those with >200 repeats were flagged by both an expanded stutter pattern and corresponding pile-up peak (**Figure 1C**).

Table 1. Correlation of Phenotype to Genotype with Assay Sizing Ranges. Table adapted from Bird et al.[3] and ACMG categorical guidelines.[4] Note EU Guidelines published by Kamsteeg et al.[1] suggest slightly different ranges than described below.

	Clinical Signs	*DMPK* Expansion	CTG Repeat Size[†]	Age of Onset	Average Age at Death
Normal	None	Stable	5 to 34	NA	NA
Premutation	None, risk to offspring	Mildly Unstable	35 to 50	NA	NA
Mild	-Cataract -Mild myotonia	Unstable, Anticipation	51 to ~150	20 to 70 yrs	60 yrs to Normal
Classic	-Weakness -Myotonia -Cataracts -Balding -Cardiac arrhythmia	Unstable within/ across tissues, Anticipation	~100 to ~1000	10 to 30 yrs	48 to 55 yrs
Congenital	-Infantile hypotonia -Respiratory deficits -Intellectual disability -Classic signs present in adults	Unstable within/ across tissues, Anticipation	>1000[‡]	Birth to 10 yrs	45 yrs[§]



[†]CTG repeat sizes are known to overlap between phenotypes. The premutation threshold has been published from 35 to 37 repeats.
‡A few individuals with congenital DM1 have been reported to have between 730 and 1000 repeats[5].
§Does not include neonatal deaths.

PCR/AGE

Genomic DNA (10-80 ng) from samples with expansions larger than 50 repeats identified previously by SB (**Figure 1B**) were PCR amplified using an evaluation version of the Asuragen AmplideX® PCR/CE *DMPK* Kit* (RUO) reagents (gene-specific primers only) and resolved by AGE following the kit protocol[7]. Briefly, the 2-primer assay (**Figure 1D**) was used for amplification. The products or a 10-fold dilution of 2-Log DNA Ladder (NEB) (10 µL total) were mixed with 6X loading dye (2 µL) and resolved on a 12-well Reliant™ Precast 1% Seakem Gold DNA MiniGel (Lonza™). Gels were imaged by visual comparison and fragment sizes were determined by comparison to the 2-log DNA ladder bands.

E-Gel™ Agarose Gels (Invitrogen) were tested as an alternate option to the Reliant gels following the same protocol outlined above. Genomic DNA from five expanded cell lines (Coriell Cell Repository) reported by Kalman et al.[6] were amplified in eight separate replicates and pooled. Either 10 µL or 5 µL were loaded onto each gel. The gels tested were 0.8% and 1.2% General Purpose (ethidium bromide), 1.2% General Purpose (SYBR Safe), and 1% EX (SYBR Gold II). Results were compared to 1% Reliant (ethidium bromide) gels using similar exposure settings. The EX gel was resolved for 13 mins, the General Purpose and SYBR Safe gels were resolved in 30 mins, and the Reliant gel was resolved for 97 mins.

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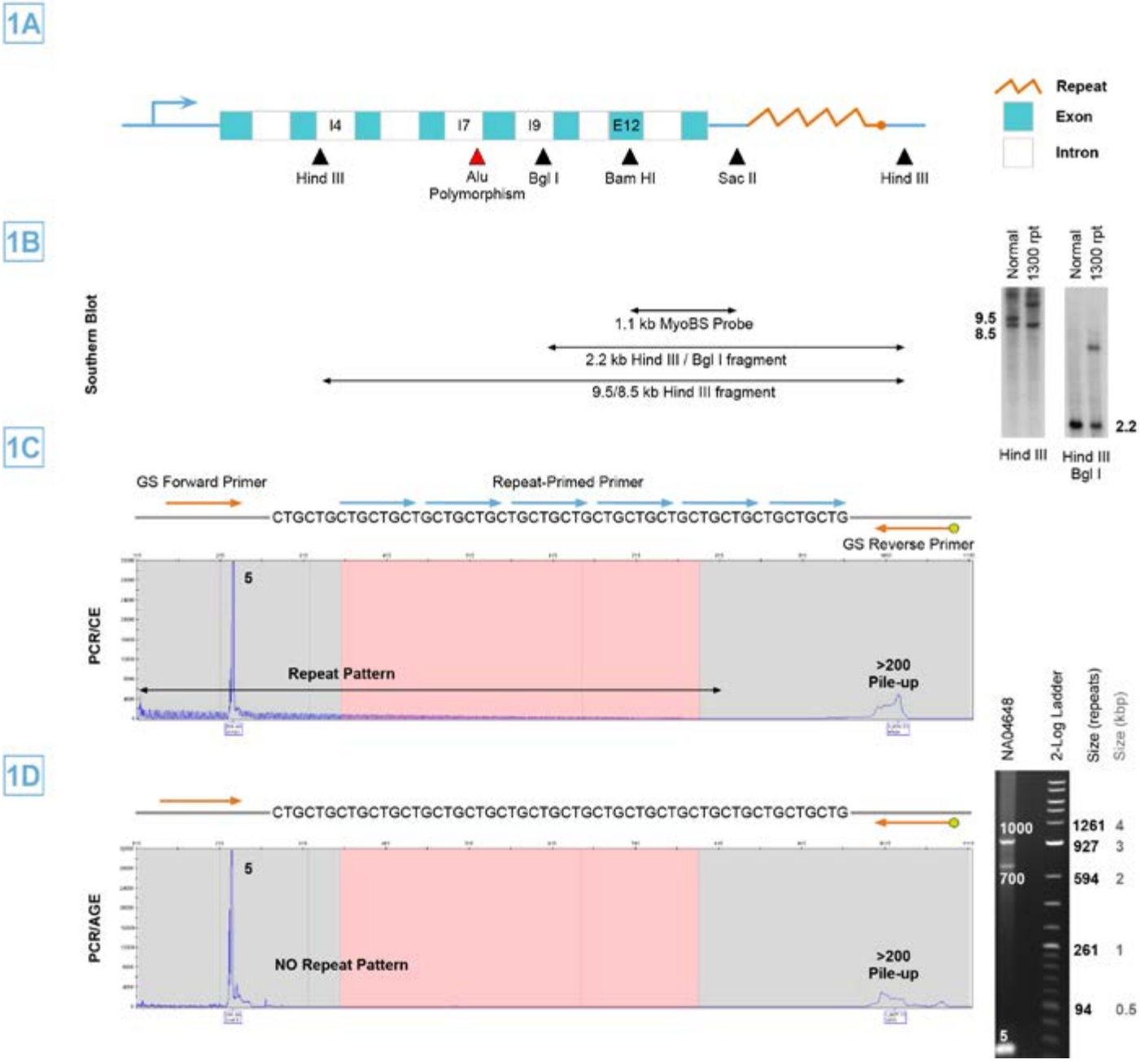

Figure 1. Assay Design. A) *DMPK* gene map with an expansion of the trinucleotide repeat CTG in the 3' UTR of *DMPK* located at Ch 19q13.3 shown. **B)** The traditional method for detecting long expansions is Southern blot. Multiple restriction digests are used to resolve shorter and longer alleles. **C)** The assay consists of two gene-specific (GS) primers (FAM-labeled reverse primer), and one repeat primer (RP) that can hybridize and prime anywhere in the repeat region. The assay produces two complementary data outputs in a single-tube: GS amplicons provide sizing of normal and expanded alleles (CE and AGE), whereas short and long alleles are either enumerated by RP stutter patterns or flagged by an aggregated "Pile-up" peak observed beyond the linear resolution of the polymer (>950bp; CE only). **D)** When only the GS primers are utilized during amplification, long alleles (>200 CTG repeats) can still be flagged by the Pile-up peak (CE) and large amplicons cab be resolved for sizing by AGE.

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RESULTS

PCR/AGE and SB Demonstrate Good Agreement Across 10 Representative Samples

The PCR/AGE assay provides optional repeat sizing beyond 200 repeats. A selection of 10 representative samples was chosen to highlight the differences in band appearance between SB and AGE (**Figure 2**). The SB comparator method relies on a restriction digest and utilizes 10 ug gDNA per reaction to size alleles without PCR amplification. Sizing is determined across the expanded range from 50 repeats to well over 2000 repeats as described in **Figure 1A & B**. For the kit, as little as 20 ng gDNA per reaction can be used for gene-specific PCR/AGE for expansions that are previously flagged by PCR/CE.

There are three typical banding patterns observed with the PCR/AGE assay:

1. Identification of ≥1 well-defined band(s) representing samples with no to low mosaicism (**Figure 2C;** S-02, S-06, S-07, S-08, S-09). These results are consistent with SB for the majority of samples up to 1000 repeats, and detection was observed up to 1900 (S-08 for example). Alleles with ≥2000 repeats did not reveal bands with the expected size using PCR/AGE though they are flagged as expanded by PCR/CE (unlike a normal sample).
2. Resolved "smears" on the PCR/AGE assay, reflecting samples with medium to high mosaicism by SB (**Figure 2C;** S-01, S-04, S-05), though the allele can still be sized based on visual inspection of the brighter portion as denoted by yellow horizontal lines in **Figure 2C**.
3. Broad, unresolved smears by PCR/AGE that cannot be easily interpreted, reflecting samples with high mosaicism by SB (**Figure 2C;** S-03, S-10).

In general, mosaic smeared bands observed by SB were more pronounced in PCR/AGE regardless of size.

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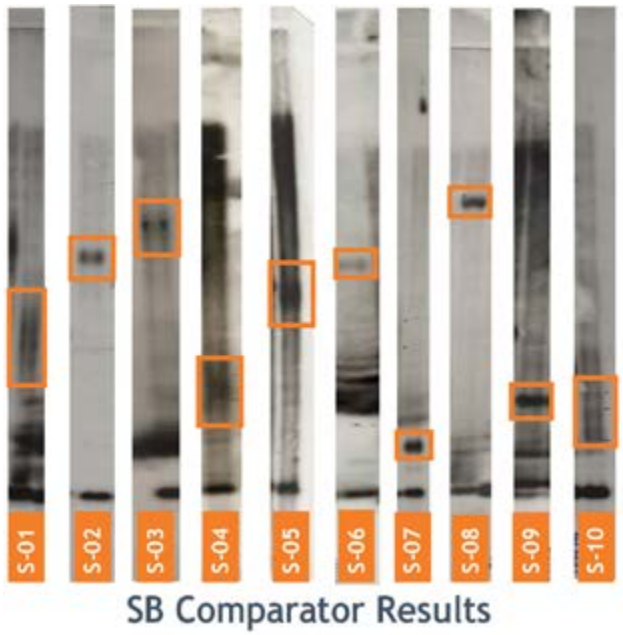
2A

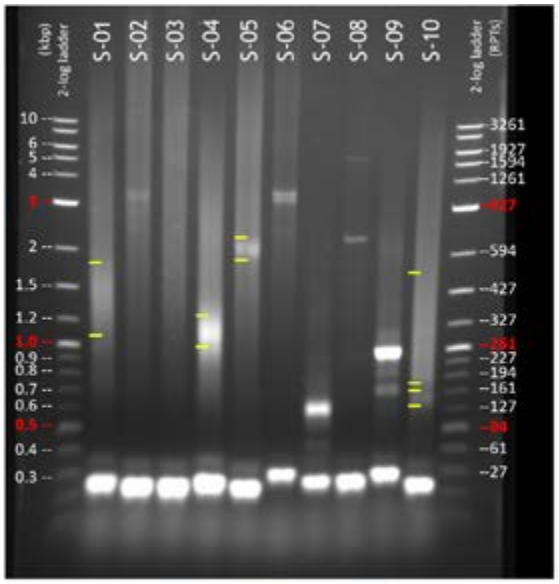
Sample Name	Site 2 SB Comparator	AmplideX PCR/CE	AmplideX PCR/AGE
S-01	11, 300-500	11, >200	Smear, High Mosaic 300-500
S-02	5, 1200-1350	5, >200	~1100
S-03	5, 1100-1500	5, >200	Smear, High Mosaic >500
S-04	12, 200-300	12, >200	~260-350
S-05	5, 800-900	5, >200	~650-700
S-06	26, 1050	26, >200	~1000
S-07	13, 100-150	13, 71, 129	70, 130
S-08	12, 1900-2220	12, >200	700, 1900
S-09	22, 300	22, >200	Mosaic, 261
S-10	5, 50-250	5, 141, >200	150, Smear 200-500

2B



SB Comparator Results

2C



Site 1 AGE Results

Figure 2. PCR/AGE and SB Comparison for 10 Representative Samples. A) Representative set of samples with sizing by SB, PCR/CE and PCR/AGE. Sizing of repeat expansions using PCR/AGE were consistent with SB for the majority of samples with low mosaicism (e.g. S-02, S-05, S-06, S-07, S-08, S-09). Sizing information is listed in CTG repeats (RPTs). **B and C)** Samples with mosaicism by SB appeared as a smear on the PCR/AGE assay (e.g. S-01, S-03, S-04, S-10) and in some cases could not easily be interpreted by AGE (e.g. S-03, S-10). Yellow lines indicate sizing of banding patterns.

PCR/AGE Categorical Agreement with Comparator SB Method for 59 Samples with >50 CTGs.

Within the collection of 67 samples, 3/67 were premutation alleles (35-50 repeats) and therefore not sized by AGE. The remaining 64 samples were evaluated by PCR/AGE. Of these, 5/64 samples failed PCR due to problems with plate seals and were not repeated for resolution by AGE (repeat analysis by PCR/CE did show that all 5 samples were expanded). The remaining 59 sample results were divided into three categories based on the smallest expanded allele size identified by SB (**Figure 3**) and summarized in **Table 2**.

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Category 1: <1000 CTGs: n=27 samples with 170-900 repeats by SB.

a. 22 of 27 samples (82%) sized by PCR/AGE were within 10% of the SB comparator size.

b. 1 of 27 samples (**Figure 3;** S-05) was sized by PCR/AGE within 20% of the SB comparator (650-700 vs 800-900 repeats respectively).

c. 1 of 27 samples (**Figure 3;** S-37) was sized with an expanded allele of 164 repeats by CE without a pile-up peak and 160 repeats by AGE. This allele was >20% different in repeat number from the 250 repeats reported by SB.

d. 3 of 27 (11%) were unresolved, smeared AGE bands (**Figure 3;** S-19, S-39, S-53) though the SB could also be interpreted as a mosaic range of repeats (*SB inset).

Category 2: 1000 up to 2000 CTGs: n=29 samples with 1000-1900 repeats by SB.

a. 20 of 29 samples (69%) sized by PCR/AGE were within 10% of the SB comparator. One of these samples (S-12) had an additional mosaic allele with >2000 repeats that was not identified by AGE (not included Category 3). Another sample (S-08) was identified as a mosaic 1900-2200 by SB and we were able to identify a discrete 1900 repeat band and a second 700 repeat band by PCR/AGE.

b. 2 of 29 samples showed PCR/AGE products within 20% of the comparator (Figure 3 S-54 and S66). The expanded allele for S-54 was sized at 1000 repeats by SB, whereas a 700 repeat band was detected by PCR/AGE. The expanded allele for S-66 was sized at 1450 repeats by SB, whereas mosaic 400 and 1000 repeat bands were identified by AGE.

c. 7 of 29 samples (24%) were unresolved, smeared AGE bands. Of these, 3/7 expanded alleles were originally sized in a mosaic size range in the comparator SB (Figure 2; S-03 and Figure 4; S-23, S-26). In 4/7 instances, (Figure 4; S-41, S-42, S-45, S-49) the allele was originally sized as a single band by SB, however, the SB morphology could be interpreted with mosaic distribution.

Category 3: ≥2000 CTGs: n=3 samples

a. 0 of 3 samples (0%) with an allele greater than or equal to 2000 repeats by SB were identified as a band or smear by AGE (S-11, S-16, and S-17). In all cases the normal allele could be observed at the bottom of the lane; however, the lane appeared similar to a homozygous normal by AGE and was flagged as expanded by PCR/CE.

Broad amplicon size distributions revealed by PCR/AGE are more prevalent in the 1000-2000 repeat range. Therefore, it is useful to compare these results to SB traces to facilitate interpretation. For instance, S-26 (1200-1700 repeats by SB) was identified as a mosaic by SB whereas S-49 (1500 repeats by SB) and S-50 (1500 repeats by SB) showed discrete sizes by SB (**Figure 4**). S-26 and S-49 appear mosaic in the scanned image and both are unresolved smears by PCR/AGE whereas S-50 appears as a discrete band by both SB and PCR/AGE (**Figure 4**). Likewise, S-52 (1150 by SB) was originally called as discrete size by SB whereas S-22 (1100-1200) was identified as a mosaic by SB though the bands appear consistent with each other in SB and both appear as a discrete band by PCR/AGE (**Figure 4**; marked by yellow lines).

To summarize, when a discrete band corresponding to <2000 repeats was observed by SB, an AGE band was typically observed within the same expanded category as defined above, often within 10% of the expected size. However, when a mosaic allele with a range ≤300 repeats was observed by SB, (e.g. S-02, S-13, S-22, S-28, S-32), a smear was typically observed by PCR/AGE, although mosaic smeared bands observed were more diffuse and harder to detect than by SB regardless of size category. When the mosaic range was larger

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(≥350 repeats, i.e. S-19, S-03, S-23, S-26), an unresolved smear was typically observed by PCR/AGE. In certain samples, a discrete size was reported based on SB gel observation yet an unresolved smear was observed by PCR/AGE (i.e. S-39, S-41, S-42, S-45, S-49, S-53). In each case, upon further analysis, the SB could be interpreted as a mosaic highlighting the complications comparing SB size reports to PCR/AGE gel data.

Table 2. Summary of Categorized Results.

Category	Category Size	Total	≤10%	Between 10-20%	≥20%	Unresolved
1	<1000 CTGs	27/59	22/27 (82%)	1/27 (4%)	1/27 (4%)	3/27 (11%)
2	1000 up to 2000 CTGs	29/59	20/29 (69%)	2/29 (7%)	0/20 (0%)	7/29 (24%)
3	≥2000	3/59	Undetected			

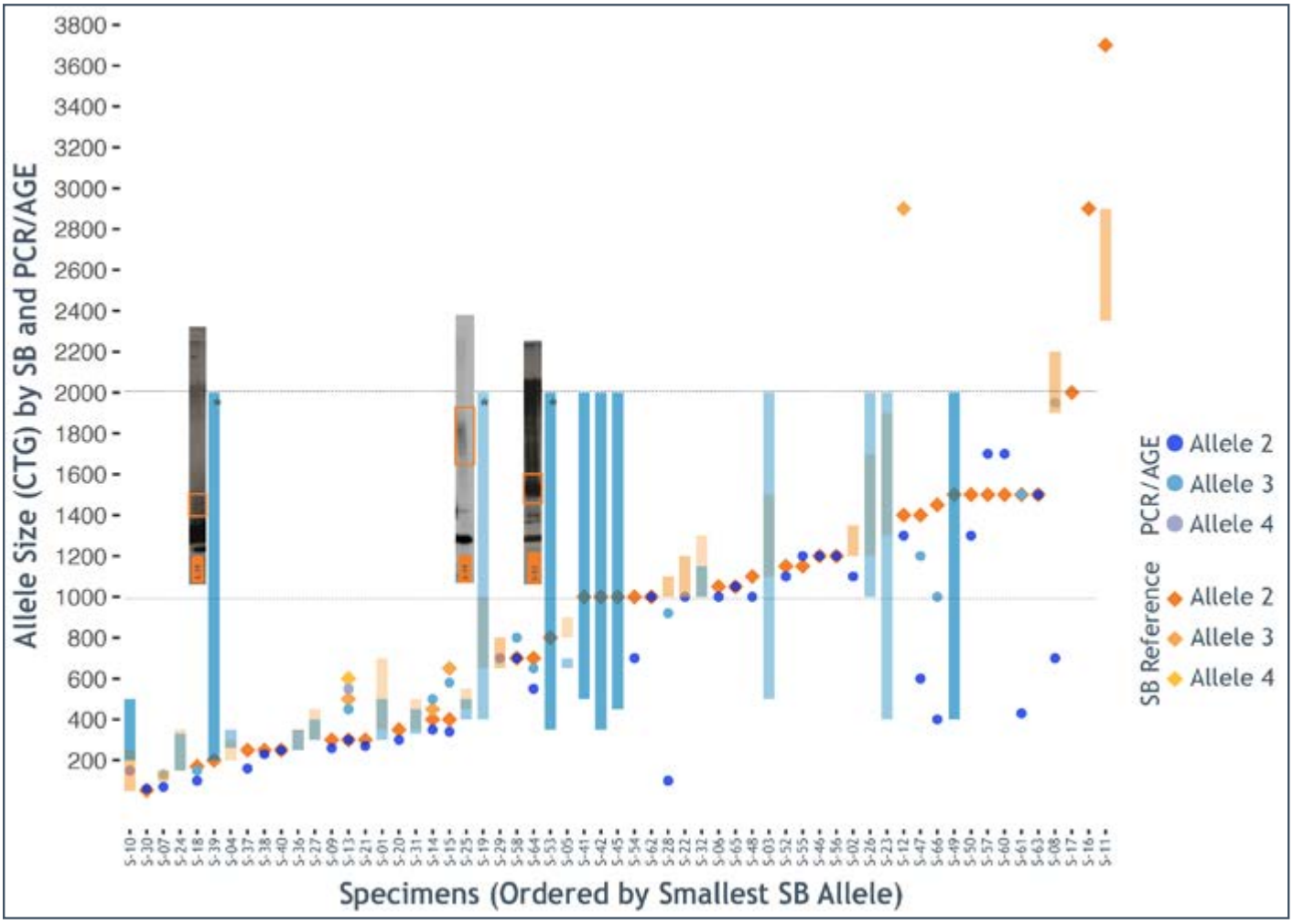

Figure 3. PCR/AGE Categorical Agreement with Comparator SB Method for 59 Samples with >50 CTGs. Samples without a blue (PCR/AGE) data point are hidden by the overlapping SB data point. Category (<1000 CTGs): 22 of 27 samples (82%) sized by PCR/AGE were ≤10% of the comparator repeat size, 1 was ≤20% (S-05), 1 was >20% (S-37), and 3 (11%) were unresolved smeared AGE bands wherein the SB could also be interpreted as a smear (* SB inset). (1000-2000 CTGs): 20 of 29 samples (69%) sized by PCR/AGE were within 10% of the comparator and 2 more were within 20%. Unresolved smeared AGE bands were observed in 7 of 29 samples (24%), and 3 were also smears by SB. (>2000 CTGs): No reference alleles could be identified by PCR/AGE (5/5), though 2 samples (S-12, S-08) with mosaic alleles in the 1000-2000 category were identified. PCR failed for 5/67 samples. Only PCR/CE was repeated for these samples and 100% were successfully flagged with >200 CTGs.

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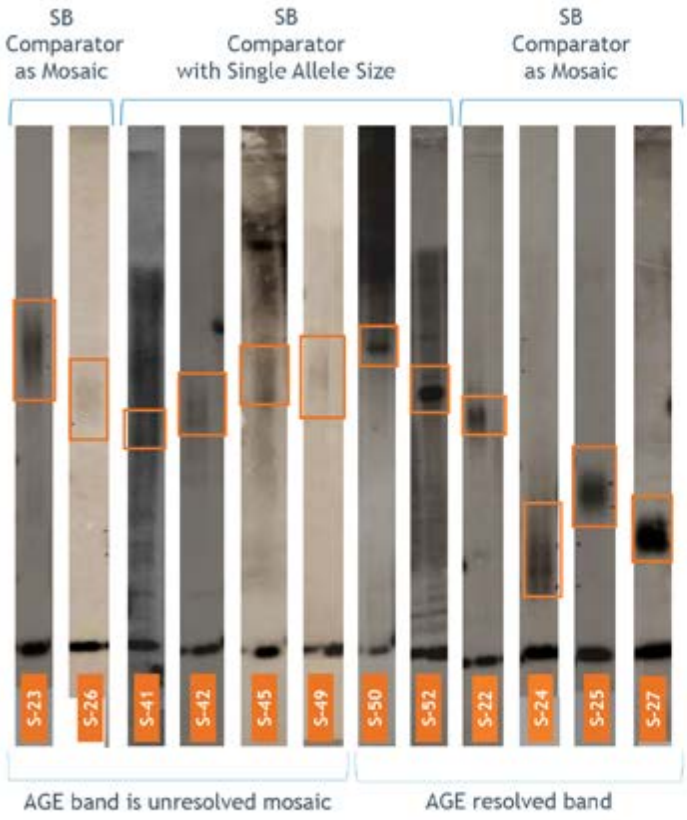

Specimen	SB Comparator (in repeats)	PCR/AGE (in repeats)
S-24	150-350	150-330
S-25	450-550	400-500
S-27	300-450	300-400
S-22	1000-1200	1000
S-23	1300-1900	SMEAR >350
S-26	1200-1700	SMEAR >350
S-41	1000	SMEAR >500
S-42	1000	SMEAR >350
S-45	1000	SMEAR >450
S-49	1500	SMEAR >400
S-50	1500	1300
S-52	1150	1100

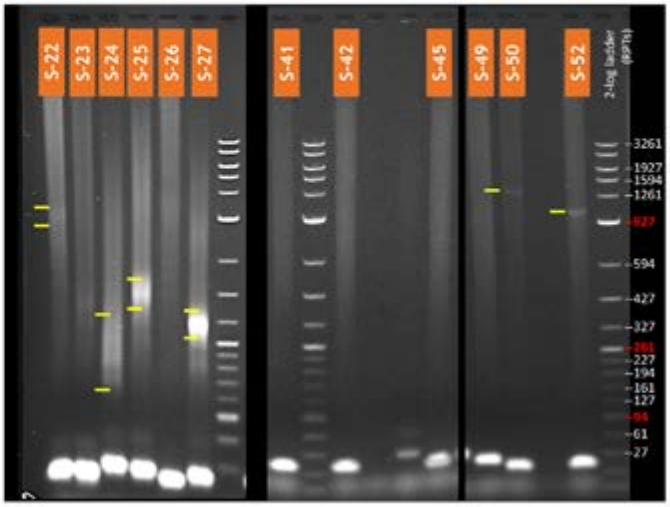

Figure 4. Selected PCR/AGE Results Compared to SB. Sizes are listed in repeats. Within the <1000 CTG category (i.e. S-24, S-25, S-27), mosaic samples were more easily resolved by PCR/AGE compared to repeat expansions outside this category. Within the 1000-2000 category, samples identified as mosaic and reported as a range by SB were more difficult to resolve by PCR/AGE (i.e. S-22, S-23, S-26). For certain samples, (i.e. S-41, S-42, S-45 and S-49) the allele was originally reported as a single size by SB, however the band morphology in the SB suggests the alleles could be a mosaic range. In each of these instances, there was an unresolved smear by PCR/AGE. This is compared to SB traces (S-50 and S-52), which were also reported as a discrete size based on SB, and a discrete band is observed in both the comparator SB and the AGE. Band sizing is subjective for both SB and PCR/AGE. In addition, the original band may be easier to identify on the original SB film compared to the scanned copy presented here. Yellow lines indicate sizing of banding patterns.

Comparison of Precast Reliant and E-gels

A comparison of precast gels was also examined. The 1% SeaKem agarose gels (Reliant) were chosen based on their superior resolution, though the gel and trays were fragile if not handled as recommended by the manufacturer. Alternatively, E-gels were tested because of faster run time and simpler protocol.

Cell-line DNA were amplified as described above, and pooled and aliquoted to reduce within sample amplification variation. The expected allele sizes are described in **Table 3** and the Reliant gel is shown in **Figure 5**. For gels pre-stained with SYBR™, the SYBR excitation (blue UV light source) and emission filters (orange camera lens filter) required longer exposure time than the corresponding ethidium filters (data not shown). SYBR Safe was not as sensitive in these gels based on the longer exposure to achieve a similar band intensity (**Figure 6A** vs **6B**). The longer exposure resulted in more background signal from the gel. Therefore, the gels could not resolve all the bands compared to ethidium bromide (**Figure 6A** vs **B;** e.g. lane 3, 700 repeat band). In addition, the E-gels could not resolve certain bands compared to the Reliant gels with ethidium

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bromide (**Figure 5** vs **Figure 6A;** e.g. lane 7). Bands from samples with only 5 µL loaded onto the gels were easily detected with Reliant gels vs E-gels (**Figure 5** vs **Figure 6A**; e.g. lanes 9-11).

The 0.8% gel resulted in bands that were more smeared as compared to the 1.2% gels, likely due to the larger pore size in the gel (**Figure 6A** vs **C;** e.g. lane 5), especially at the smaller band sizes between 100 and 250 repeats. It was difficult to determine where the brighter ladder bands at 928, 261, and 94 repeats were located on the gel. Depending on the allele size, the gel could be run at different times based on the Categories outlined above to resolve <1000 or 1000 up to 2000). All of the general-purpose gels also showed a characteristic "U" pattern across the gel where samples in the center of the gel traversed faster and thus sizing with a ladder at the edges was more difficult compared to Reliant gels.

The 1.0% EX™ gels pre-stained with SYBR Gold II provided the best band intensity to background of any gel and displayed sharper band resolution (**Figure 6A** vs **D**, e.g. lane 3). These gels likely are made with a different agarose than the GP gels. The stain or agarose consistency also interfered with ladder resolution, especially >1000 and there was a detectable smear in the center of each lane that was not present in the other gels.

Table 3. Expected Expanded Allele Sizing Across Five Different Cell Lines Sourced from Coriell Cell Repository. Sample NA04648, NA03696, and NA05152 were sized by three independent labs as identified by Kalman et al.[6] Sizes underlined are major (bright) bands by PCR/AGE. All sizes listed in CTG repeats.

Sample	Expected Allele	Observed Size by PCR/AGE
NA04034	700	200, <u>600</u>
NA04648	1008±49	600, <u>1000</u>
NA23256	160-170, 775	100, 125, <u>260</u>, <u>430</u>, 700
NA03696	697±13	400, <u>450</u>, 600
NA05152	1621±30	1500

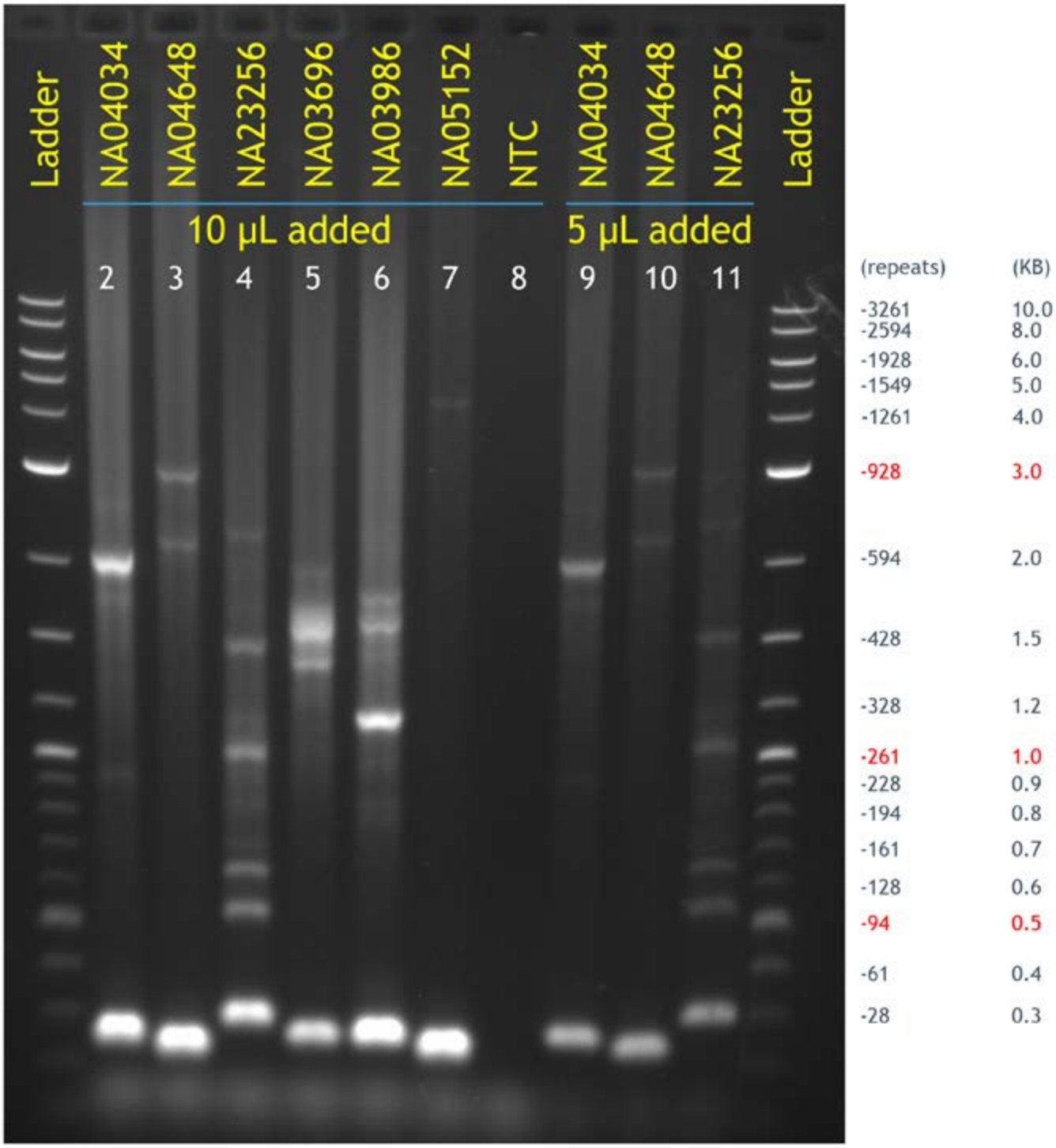

Figure 5. Reliant 1.0% Seakem Agarose Gel (Ethidium Bromide) Resolved for 100 Mins, exposed for 300ms.

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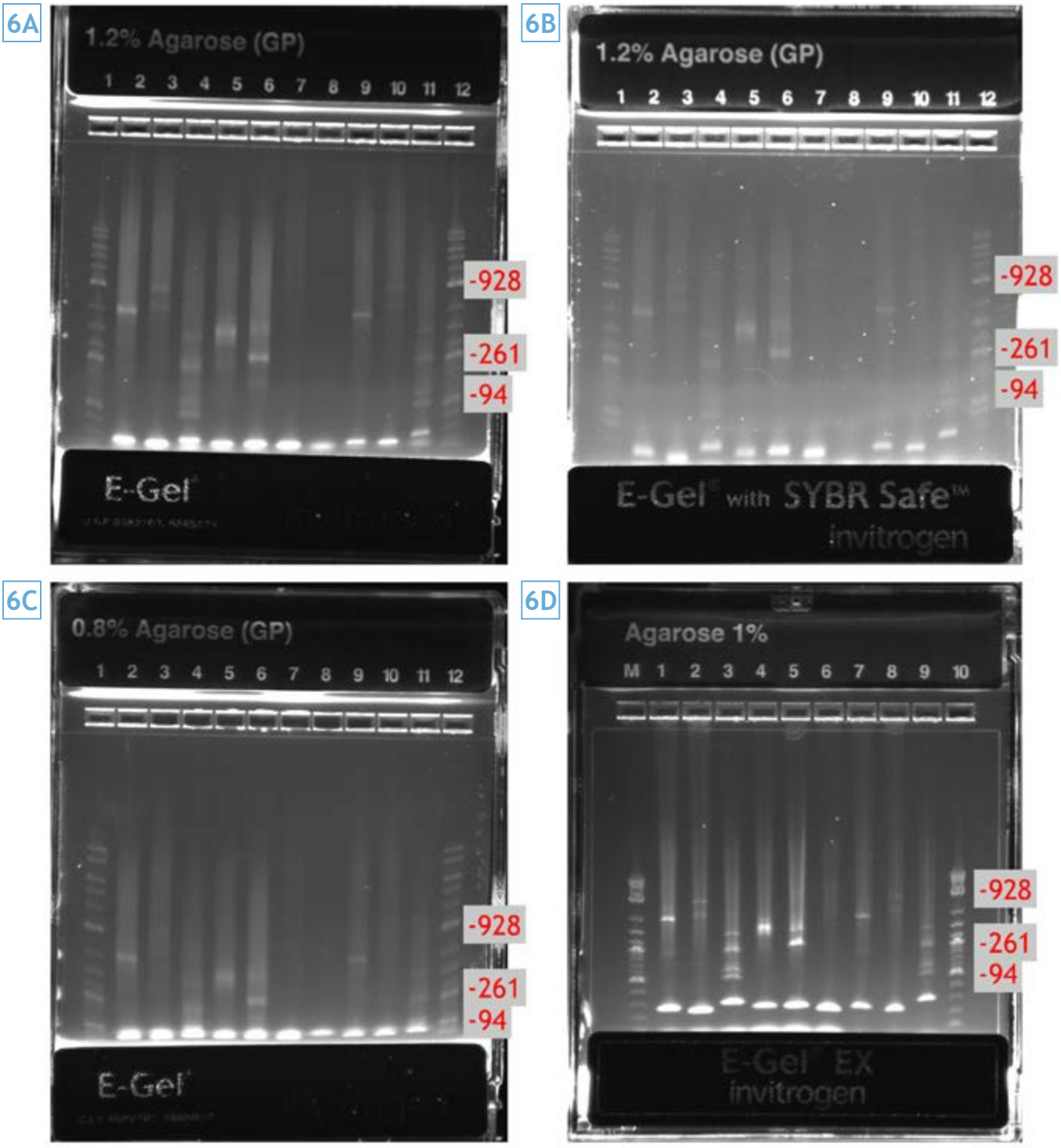

Figure 6. E-gel Images for Each of the Samples. A) 1.2% General Purpose (ethidium bromide), resolved for 30 mins, exposed for 600ms. **B)** 1.2% General Purpose (SYBR Safe) resolved for 30 mins, exposed for 1000ms **C)** 0.8% General Purpose (ethidium bromide) resolved for 30 mins exposed for 400ms **D)** 1.0% EX (SYBR Gold II) resolved for 13 mins, exposed for 200ms.

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SUMMARY AND CONCLUSIONS

- In each sample with an expanded allele >200 repeats identified by SB, PCR/CE correctly flagged the expansion from the pile-up and repeat-primed peak patterns.
- The PCR/AGE assay demonstrated consistent size agreement with SB analysis up to 1000 repeats (82% were sized within 10% of comparator and 85% were sized within 20% of the comparator).
- The PCR/AGE assay demonstrated less consistent size agreement with SB analysis from 1000 up to 2000 repeats (69% were sized within 10% of comparator and 76% were sized within 20% of comparator).
- In this study, the largest allele detected and sized by PCR/AGE consistent with SB was 1900 repeats.
- Smeared bands appeared more diffuse on AGE after PCR amplification than the comparator restriction-digested band resolved by SB.
- E-Gels are not recommended for use with the PCR/AGE workflow due to poor sensitivity and resolution compared to Reliant gels.
- The AmplideX PCR/CE/AGE workflow can reduce the labor and turnaround time from sample to answer compared to SB analysis. For samples with >1900 repeats or an unresolved AGE smear, SB may be required to obtain an accurate repeat size.

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