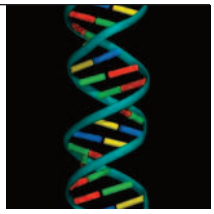
A644: A multi-oligo control covering all mutations tested in the Luminex CF assay



The ACCURUN® 644 Cystic Fibrosis 40+4 Control is a synthetic oligonucleotide control, designed and validated on the Luminex xTag™ CFTR assay. Recently, Luminex updated and replaced their cystic fibrosis testing platform, replacing the xTag™ CFTR assay with the new xTag™ Cystic Fibrosis 39 version 2 assay (CF39v2). The CFTR assay is no longer available.

This Technical Bulletin explains the interactions between the ACCURUN 644 Control and the test results when using this control on the new Luminex xTag™ CF39v2 test platform. The CF39v2 test platform has a different software suite which assigns mutational calls, with call reporting now based on a 2 allele premise. There are no changes to the mutations tested or detected between the CFTR and CF39v2 assays, but there are differences in how the software interprets raw data to make zygosity calls for each mutation.

Possible calls include **HET**, **Mu D**, **Wt D**, and **No Call**, as well as **CH** or individual results for reflex mutations. Crossover studies indicate that the ACCURUN 644 Control can still be utilized on the new xTag™ CF39v2 kit, even though there are some changes to the results on the new platform. As has always been recommended, inspection of the Allelic Ratios and Raw Signals is needed, in addition to examination of the calls reported, for a complete picture of control and assay performance.

What is an oligo control?

ACCURUN 644 is part of a family of genetic controls for cystic fibrosis made by SeraCare which are composed of synthetic oligonucleotides (oligos) in solution. Each oligo is a short piece of DNA, containing one or more mutations and flanking regions (see Figure 1, page 2). Every oligo is capped at both ends with gene sequences corresponding to control-specific primers used in the amplification step of the assay. The corresponding primers are also included in the control mixture. The ACCURUN 644 Control is comprised of multiple oligos to provide full coverage for all the mutations tested on both the CFTR and CF39v2 assays. (See Figure 2, page 3).

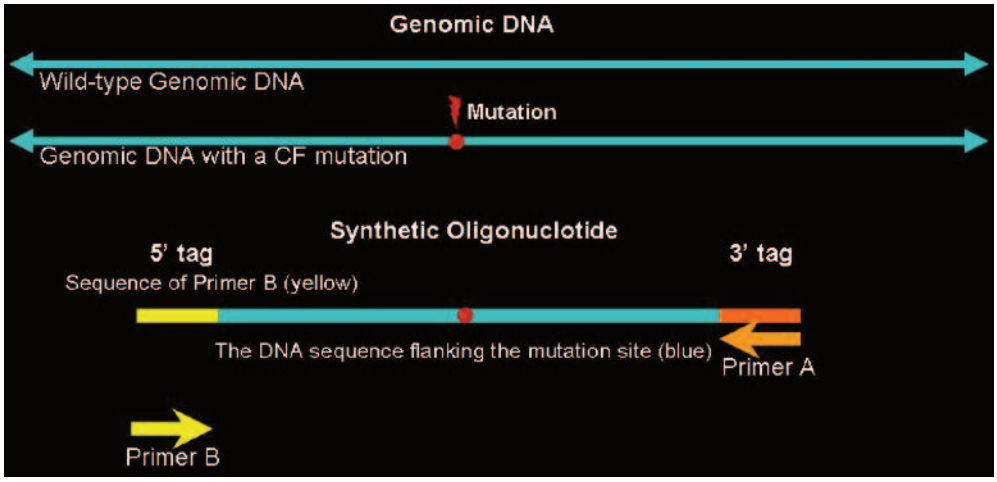
> Adding the ACCURUN 644 Control to your QC scheme offers significant advantages over the use of rotating genomic DNA QC scenarios because it allows coverage of all mutations in a single test run.

The ACCURUN 644 Control is designed to examine the detection step of the assay, not the amplification step. The control employs different primers than the platform uses on patient samples; therefore primer binding and extension steps in a control run do not represent primer binding and extension in a genomic patient sample.

Given that oligo controls only monitor the detection step of the Luminex assay, it is also important to run an appropriate genomic DNA control and a "no template" control to ensure that every aspect of the testing platform is challenged. Mandatory positive and negative controls must be run in addition to ACCURUN controls.

Adding the ACCURUN 644 Control to your QC scheme offers significant advantages over the use of rotating genomic DNA QC scenarios because it

Figure 1. Design of synthetic oligos



Each oligo covers one or more mutations and a small flanking sequence of DNA, enough to allow the platform to use these short pieces of DNA to detect the mutation. Each end is capped by a sequence corresponding to a primer that is used in the amplification step to amplify the oligo. The control is comprised of many oligos corresponding to the detected mutations as well as primer A and primer B used in the amplification of the entire assembly of oligos.

allows coverage of all mutations in a single test run. Genomic DNA does not provide comprehensive coverage for the detection of every mutation, and multiple tests must be run to gain an understanding of assay performance over even a small number of mutations.

The Two Allele Premise

In the update from CFTR to CF39v2, Luminex has updated the software package used with the assay from TDAS CF-1 1.11 to the new TDAS CFTR 2.00. The new software program bases zygosity calls for each mutation on a two allele premise. This change may affect the calls made by the software when using ACCURUN 644, which contains significantly more than two alleles.

The two allele premise of the TDAS CFTR 2.00 means the software is programmed with algorithms exclusively allowing results consistent with patient samples which have only 2 copies of the gene. As the assay detects the amplified regions of the gene, the ratio between a mutant and wild type version should always be consistent with the presence of two alleles, reading one of the following results:

- two wild type (Wt D)
- two mutant (Mu D)
- one of each (HET)

As such, there are three allelic ratio (AR) thresholds set into the software algorithm for each mutation. These are listed on your data report as AR thresholds (See Figure 3, page 5):

- wild type call threshold (WT Call)
- wild type present (Wt Present)
- mutant present (Mut Present)

Using these AR thresholds, the software dictates that for a positive heterozygous result (HET call), for instance, the following conditions must be met:

1. The mutant allelic ratio (Mut Allele) must exceed the Mut Present AR threshold indicating the presence of the mutant allele.

2. The wild type allelic ratio (Wt Allele) must fall

below the WT Call AR threshold and above the Wt Present AR threshold, indicating the presence of the wild type allele, but also indicating that allele is not present in two copies.

If these conditions are met, the software will generate a positive heterozygous result (HET Call). However, if the Wt D Allele and the Mut Allele both fall below their respective Wt Present and Mut Present AR thresholds, the software will generate a No Call.

AR thresholds vary from mutation to mutation. Luminex has accounted for variation in both the

Figure 2. Expected Calls for ACCURUN 644 using Luminex assay CF39v2

Since the Allelic Ratio can change from run to run in a way that affects the interpretation of the call, some mutations may have more than one acceptable call. The examination of net signal and Allelic Ratio is critical to understanding the performance of the assay and control. Calls changing from run to run and in some instances the existence of no calls should not result in control run failure.

Variation	Call
G85E	Mu D or HET
394delTT	HET or No Call [1]
R117H	HET
Y122X	HET
621+1G>T	Mu D
711+1G>T	Mu D
1078delT	Mu D
R334W	Mu D
R347P	Mu D
R347H	Mu D
A455E	Mu D
Δl507	Wt D or HET [2]
ΔF508	HET [2]
V520F	Mu D
1717-1G>A	Mu D

Variation	Call
G542X	HET
S549N	HET
S549R(T>G)	HET
G551D	No Call [3]
R553X	HET
A559T	Mu D
R560T	HET
1898+1G>A	Mu D
1898+5G>T	HET
2183AA>G	Mu D or No Call [4]
2184delA	Mu D
2307insA	Mu D
2789+5G>A	Mu D
3120+1G>A	Mu D

Variation	Call
Y1092X-C>G	Mu D
Y1092X-C>A	Mu D
M1101K	Mu D
R1162X	Mu D
3659delC	Mu D or HET
S1255X(ex 19)	Mu D
S1255X(ex20)	HET
3849+10kbC>T	Mu D
3876delA	Mu D or HET
3905insT	Mu D or HET
W1282X	Mu D
N1303K	Mu D
5T/7T/9T	5T/9T D [5]
I506V/I507V/F508C	CH [6]

Data Investigation Steps

1. In the event of a no call, examine the Raw Signals and Allelic Ratios. The run should not be considered a failure if the Raw Signals and Allelic Ratios for the 394delTT mutation are similar in strength and ratio to other heterozygous calls in the run.
2. In the event of Wt D Call, examine the Raw Signals and Allelic Ratios. The run should not be considered a failure if the software is still detecting a readable signal for ΔI507, even if lower than the 0.30 AR threshold that results in a HET call.
3. The G551D mutation is always reported as No Call, even when the signal strength and Allelic Ratios are within the normal, expected range due to a software algorithm that occludes the G551D Mu D or HET calls when the R553X is not Wt D. Raw Signals and Allelic Ratios should be examined to ensure that in the absence of this algorithm, a HET call would have been made (Mut Allele exceeds Mut Present AR threshold of 0.25 with net signals close to those of other mutations).
4. In the event of a No Call, examine the Raw Signals and Allelic Ratios. A No Call could be a result of the Mut and Wt Allelic Ratios both not reaching the AR thresholds. The run should not be considered a failure if the platform is detecting the 2183AA>G but not meeting the 0.35 Mut Present AR threshold.
5. 7T may read as wild type, but raw data should indicate a signal for 7T is present.
6. CH (call hidden) will be reported if theΔI507 and ΔF508 Calls are not Mu D. Since those mutations are expected be called as HET or Wt D, the I506V/I507V/F508C call will remain hidden.

amplification steps (different primers, different regions) and in the specificity of the detection for each mutation in the software algorithms.

Some AR thresholds are purposely set high to avoid false positives, while others are set low to avoid false negatives. Luminex has carefully screened their chemistries in the context of genomic DNA to determine the appropriate AR thresholds for accurate calls.

The chemistries for synthetic oligo controls are different than for genomic DNA in patient samples. Due to the close proximity of some CF mutations, there are oligos carrying more than one mutation, and overlaps occur between oligos (the same sequence is carried on more than one oligo). This could appear to the Luminex software as a non-diploid ratio of alleles.

Because oligos are synthetically manufactured, such situations can usually be avoided by adjusting the concentration of individual oligos in the control solution, thus affecting the total Allelic Ratios (total wild type signal vs. mutant signal) for the overlapping sequences carried on more than one oligo.

SeraCare designed and optimized the oligo concentrations in ACCURUN 644 for the CFTR assay, resulting in some inconsistencies in reported results when this control is used with the CF39v2 assay.

Given that synthetic oligo controls have ratios of mutant to wild type sequences that would never be found in patient samples, the Allelic Ratios may run close to the cut off between a HET Call and a Mu D Call for certain mutations.

Run-to-run variation where the call flips between HET and Mu D is not a concern as long as the Allelic Ratio shift is not significant and the signal strength has not dropped. The intention of an oligo control is to demonstrate that the assay can accurately detect every mutation. Since an oligo control does not assess the amplification steps, subtle changes in Allelic Ratio leading to significant differences in the software zygosity call (HET, Mu D, Wt D, or No Call) should not be a concern.

Examination of the Raw Signals and Allelic Ratios lead to a more complete picture of instrument performance than analysis of the calls alone. Figure 3, page 5 indicates the expected calls generated by TDAS CFTR 2.00 for a run of ACCURUN 644 when run on CF39v2.

Investigating specific calls to determine if the Luminex xTag™ CF39v2 assay is working properly

The 394delTT mutation has a particularly high AR threshold for Mut Present at 0.41 and for WT Call at 0.68 (see Figure 2, page 3). The concentration of 394delTT in ACCURUN 644 results in an Allelic Ratio that is very close to the Mut Present AR threshold.

Within normal run-to-run variation, the signal may dip below the Mut Present AR threshold. When this happens, the wild type allelic ratio will likely not exceed the WT Call AR threshold and the software will report No Call for the mutation. In the event of a no call, examine the Raw Signals and Allelic Ratios.

The run should not be considered a failure if the Raw Signals and Allelic Ratios are similar in strength and ratio to other heterozygous calls in the run.

The ΔI507 mutation is one of a small number of mutations that are examined by the software in conjunction with another nearby mutation, ΔF508. Instead of the Allelic Ratio being split between two alleles (mutant and wild type), the Allelic Ratio is now split between three alleles: ΔI507 mutant, ΔF508 mutant, and wild type. As such, the Mut Present AR Threshold at 0.30 for ΔI507 does not leave much room for run-to-run variation in an oligo based control where all 3 alleles are expected to be present. The signal for ΔI507 for ACCURUN 644 is often below the 0.30 threshold resulting in a Wt D call. In the event of Wt D Call, examine the Raw Signals and Allelic Ratios. The run should not be considered a failure if the software is still reporting a detectable signal for ΔI507, albeit too weak to result in a HET call.

The G551D mutation, when running ACCURUN 644 on the new Luminex platform, should always be reported as No Call even when the signal strength and Allelic Ratios are within the normal, expected

Figure 3. Example Data

An example run, demonstrating typical results when using ACCURUN 644 on the Luminex CF39v2 assay. Actual results may vary.

Variation	Call	Raw Signals (MFI)		Background (MFI)		Net Signals (MFI)		Allelic Ratios		AR Thresholds		
		Wt Allele	Mut Allele	Wt Allele	Mut Allele	Wt Allele	Mut Allele	Wt Allele	Mut Allele	WT Call	Wt Present	Mut Present
G85E	HET	1560.0	1492.0	20.0	44.0	1540.0	1448.0	0.52	0.48	0.80	0.27	0.30
394delTT	HET	2959.0	2366.0	22.0	20.0	2937.0	2346.0	0.56	0.44	0.68	0.40	0.41
R117H	HET	2234.0	3222.0	36.5	25.0	2197.5	3197.0	0.41	0.59	0.85	0.26	0.25
Y122X	HET	1595.5	1969.0	29.0	21.0	1566.5	1948.0	0.45	0.55	0.85	0.25	0.25
621+1G>T	Mu D	73.0	1839.5	32.0	24.0	41.0	1815.5	0.02	0.98	0.85	0.25	0.25
711+1G>T	Mu D	47.5	2028.5	29.0	22.0	18.5	2006.5	0.01	0.99	0.85	0.25	0.25
1078delT	Mu D	173.0	1709.0	25.0	34.0	148.0	1675.0	0.08	0.92	0.80	0.33	0.30
R334W	Mu D	186.0	1518.5	25.0	43.5	161.0	1475.0	0.10	0.90	0.75	0.28	0.35
R347P	Mu D	79.0	1790.0	26.0	26.0	53.0	1764.0	0.02	0.54	0.85	0.27	0.25
└ R347H	Mu D		1510.5		32.0		1478.5		0.45			0.20
A455E	Mu D	28.0	1849.0	32.0	25.0	0.0	1824.0	0.00	1.00	0.85	0.25	0.25
dI507	Wt D	545.0	651.0	11.0	35.0	534.0	616.0	0.22	0.25	0.80	0.20	0.30
└ dF508	HET		1356.0		27.0		1329.0		0.54			0.18
V520F	Mu D	50.5	2763.5	44.0	13.0	6.5	2750.5	0.00	1.00	0.85	0.25	0.25
1717-1G>A	Mu D	69.0	1747.0	11.0	21.0	58.0	1726.0	0.03	0.97	0.85	0.30	0.25
G542X	HET	3155.0	2680.0	17.5	25.0	3137.5	2655.0	0.54	0.46	0.75	0.25	0.35
S549N	HET	1673.5	3388.0	39.0	42.0	1634.5	3346.0	0.33	0.67	0.85	0.28	0.25
S549R(T>G)	HET	3028.5	1772.0	11.5	19.5	3017.0	1752.5	0.63	0.37	0.85	0.25	0.25
G551D (1)	No Call	3803.0	1680.5	30.0	31.5	3773.0	1649.0	0.70	0.30	0.85		0.25
R553X	HET	2932.5	1178.0	38.5	19.0	2894.0	1159.0	0.71	0.29	0.85	0.25	0.25
A559T	Mu D	261.0	1435.0	21.0	18.0	240.0	1417.0	0.14	0.86	0.80	0.29	0.30
R560T	HET	946.0	815.5	33.5	20.0	912.5	795.5	0.53	0.47	0.85	0.25	0.25
1898+1G>A	Mu D	176.0	1251.0	38.0	27.5	138.0	1223.5	0.10	0.90	0.85	0.25	0.25
1898+5G>T	HET	2061.0	1671.5	34.0	37.0	2027.0	1634.5	0.55	0.45	0.83	0.25	0.27
2183AA>G	Mu D	174.0	2358.5	38.0	27.0	136.0	2331.5	0.02	0.38	0.77	0.20	0.35
└ 2184delA	Mu D		3672.0		8.0		3664.0		0.60			0.20
2307insA	Mu D	171.5	3155.0	22.0	34.0	149.5	3121.0	0.05	0.95	0.80	0.30	0.30
2789+5G>A	Mu D	58.5	1654.0	34.0	39.0	24.5	1615.0	0.01	0.99	0.85	0.25	0.25
3120+1G>A	Mu D	67.5	1803.0	40.0	19.0	27.5	1784.0	0.02	0.98	0.85	0.25	0.25
Y1092X-C>G	Mu D	76.0	2134.0	38.0	19.0	38.0	2115.0	0.01	0.52	0.75	0.25	0.30
└ Y1092X-C>A	Mu D		1969.0		20.0		1949.0		0.48			0.30
M1101K	Mu D	93.0	3016.5	23.5	22.0	69.5	2994.5	0.02	0.98	0.81	0.25	0.29
R1162X	Mu D	96.0	2036.0	23.0	29.0	73.0	2007.0	0.04	0.96	0.85	0.30	0.25
3659delC	HET	1025.0	2276.0	62.0	83.5	963.0	2192.5	0.31	0.69	0.81	0.31	0.29
S1255X(ex.19)	Mu D	44.0	1170.5	37.0	29.0	7.0	1141.5	0.01	0.99	0.85	0.28	0.25
S1255X(ex.20)	HET	1984.0	3014.0	38.0	15.0	1946.0	2999.0	0.39	0.61	0.85	0.25	0.25
3849+10kbC>T	Mu D	157.0	3504.0	29.5	41.5	127.5	3462.5	0.04	0.96	0.82	0.26	0.28
3876delA	HET	1652.0	3156.0	31.0	31.0	1621.0	3125.0	0.34	0.66	0.80	0.25	0.30
3905insT	Mu D	1497.0	3138.0	45.0	19.0	1452.0	3119.0	0.32	0.68	0.80	0.35	0.30
W1282X	Mu D	160.5	2737.5	42.5	35.0	118.0	2702.5	0.04	0.96	0.85	0.25	0.25
N1303K	Mu D	55.0	3324.0	20.0	42.5	35.0	3281.5	0.01	0.99	0.85	0.25	0.25
5T	5T/9T D		3767.0		38.0		3729.0		0.48			0.30
├ 7T			1216.5		33.0		1183.5		0.15			0.30
└ 9T			2940.0		24.0		2916.0		0.37			0.25
I506V	CH				25.0							
├ I507V					28.0							
└ F508C					30.0							

1. Variation failed: Allelic Ratio(s) not within predefined ranges

range. Genomic samples with the G551D mutation run side-by-side with the oligo control show the normal expected result, indicating that there is not a concern with the platform's ability to make a call on the G551D mutation. The source of this discrepancy is a software algorithm that is triggered by the oligo control, and it is not an indication of performance failure on the control run.

The site of the G551D mutation lies within close proximity to the site of the R553X mutation - these mutations are within 5 nucleotides of each other, but unlike ΔI507 and ΔF508, which are equally close, the software examines the G551D and R553X mutation separately.

To avoid potential false readings of G551D in the presence of the R553X mutation, the software is equipped with an algorithm that occludes any non-wild type call of G551D when R553X is present. The ACCURUN 644 Control contains both the G551D and R553X mutations. Raw Signals and Allelic Ratios should be examined to ensure that in the absence of the software algorithm occluding the call, a HET call would have been made (Mut Allele AR exceeds Mut Present AR Threshold of 0.25 with net signals close to those of other mutations).

The 2183AA>G mutation, like the ΔI507 mutation, is examined by the software in conjunction with another nearby mutation, 2164delA. The signal for the 2164delA is often strong; sometimes the signal for 2164delA is so strong that the lower signals for 2183AA>G and wild type do not pass either the Mut Present or the Wt Present AR thresholds, resulting in a No Call for 2183AA>G. The run should not be considered a failure if the platform is detecting the 2183AA>G but not meeting the 0.35 Mut Present threshold.

The 5T/7T/9T mutations are also examined by the software collectively. Since the signal is split 3 ways between these mutations, there is not much margin for variation in signal. With the current formulation of the oligo control, the 7T signal is usually below the AR threshold for a 7T call. The run should not be considered a failure if the platform is detecting a 7T signal but not meeting the 0.30 Mut Present threshold.

Conclusion

The simplest examination of data on the Luminex xTag™ CF39v2 assay is inspection of the calls on any sample. For a quality control run of the platform using the ACCURUN 644, a simple review of the calls would give an incomplete picture of the performance of the platform and control. In order to be fully informed of the functionality of an assay when looking at A644 oligo control data, lab managers need to be certain to inspect the raw signal and allelic ratios.

For a clear understanding of the changing performance of the control, Figure 2, page 3, indicates the calls expected for each mutation, noting that more than one possibility exists for some mutations. SeraCare is examining the possibility of optimizing the ACCURUN Cystic Fibrosis Controls for performance on CF39v2. Until product development efforts have completed, ACCURUN 644 still provides full coverage of every mutation detected with the Luminex xTag™ CF39v2 assay in a single well, a significant advantage over rotating genomic DNA schemes or other synthetic formulas that require multiple runs to test all mutations.

About the Author

Tamara Smith is a geneticist by training and Senior Product Manager at SeraCare.

Address correspondence to tsmith@seracare.com