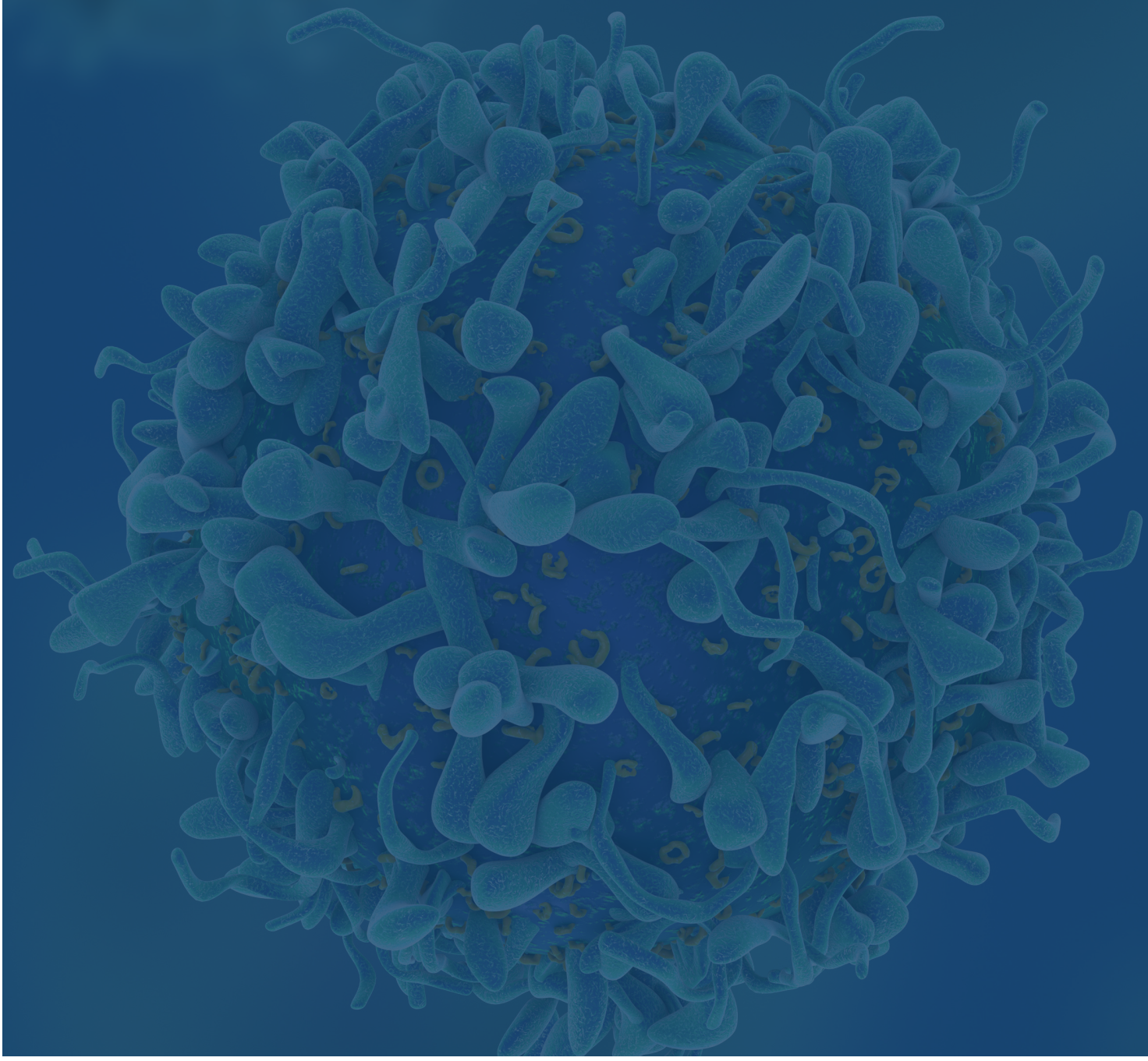
invivoscribe®

T-Cell Receptor Gamma Capillary Electrophoresis Assays

Research Use Only (RUO)



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1. Overview of Assays

Both Invivoscribe's T-Cell Receptor Gamma Gene Rearrangement Assay 2.0 and ***TCRG*** Gene Clonality Assay were designed to identify T-cell clonality in suspect lymphoproliferations and are to be used in conjunction with other laboratory tests and results interpreted in the context of all available information. Despite this similarity, there are some noteworthy differences.

The ***TCRG*** Gene Clonality Assay includes two master mixes: ***TCRG*** Tube A and ***TCRG*** Tube B. ***TCRG*** Tube A contains primers that target the Vγ1-8 + Vγ10 genes and Jγ1.1, Jγ1.3, Jγ2.1, and Jγ2.3 genes (also known as JγP1, Jγ1, JγP2, and Jγ2, respectively), generating 4 polyclonal distribution size ranges. ***TCRG*** Tube B contains primers that target the Vγ9 + Vγ11 genes and Jγ1.1, Jγ1.3, Jγ2.1, and Jγ2.3 genes, generating 4 polyclonal distribution size ranges (see figure).

Conversely, the T-Cell Receptor Gamma Gene Rearrangement Assay 2.0 includes a single master mix, ***TCRG*** – 6FAM, which targets genes described in lymphoid malignancies, including the Vγ2, 3, 4, 5, 8, 9, 10, & 11 and Jγ1/Jγ2,JγP, and JγP1/JγP2 regions. The resulting polyclonal background lies within a single valid size range, generating a more robust and easily interpreted signal in comparison to other ***TCRG*** clonality assays which have multiple valid size ranges. Moreover, this design is better suited to detect ***TCRG*** gene rearrangements in FFPE samples, due to its smaller valid size range of 159 bp to 207 bp (see figure).

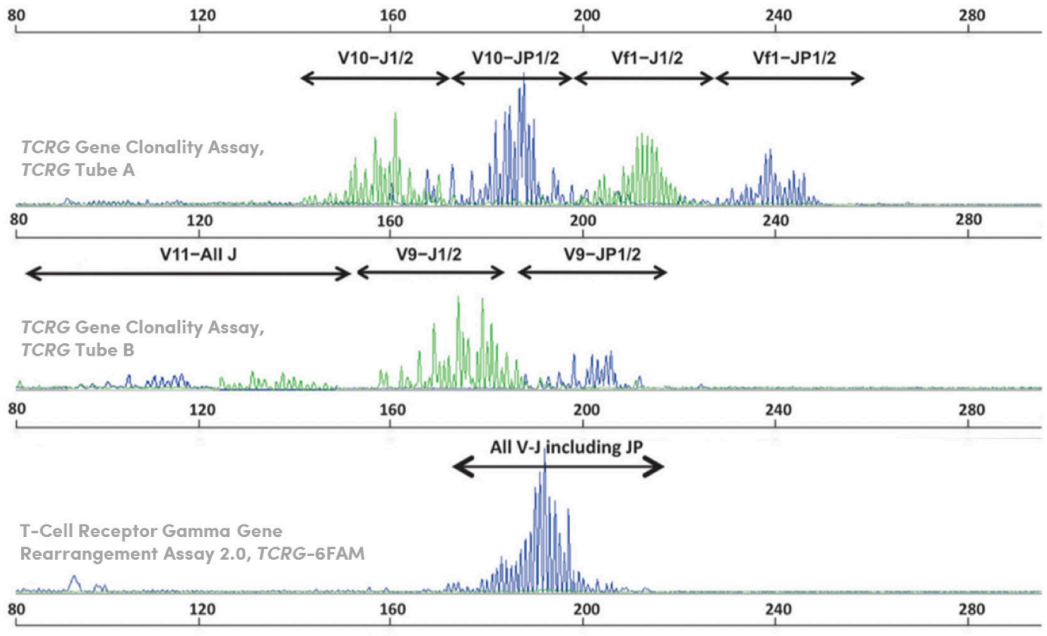
Some of the benefits of the T-Cell Receptor Gamma Gene Rearrangement 2.0 Assay include:

- Allows a higher sample throughput with the single master mix assay
- Offers easy setup and simpler interpretation than the two-tube approach
- Generates smaller amplicon sizes, meaning better success when testing FFPE samples
- Reduces the DNA required for testing by half by reducing PCR reactions from two to one

Figure 1: GeneScan analysis indicating rearrangement valid size ranges generated from the same polyclonal sample with the assays listed below.

(A) ***TCRG*** Gene Clonality Assay, ***TCRG*** Tube A master mix;
(B) ***TCRG*** Gene Clonality Assay, ***TCRG*** Tube B master mix;
(C) T-Cell Receptor Gamma Gene Rearrangement Assay 2.0, ***TCRG***-6FAM master mix

Figure adapted from: Marine, A., ***et al.,*** (2019) ***HemaSphere*** 3(3):e255.



2. Assay Performance Characteristics

These two assays were designed differently, thus the results generated with each assay are interpreted differently. The primary improvements of the T-Cell Receptor Gamma Gene Rearrangement Assay 2.0 over the ***TCRG*** Gene Clonality Assay are the addition of JγP target and consolidation of multiple polyclonal distributions into a single distribution. When PCR products are grouped into a single distribution, it generates an increased signal, improving sensitivity and reducing the likelihood of false positive from minor peaks in dispersed repertoires. For example, minor peaks in the Vγ11 valid size range can be mistakenly reported as significant due to lack of other polyclonal peaks within that distribution.

Because the T-Cell Receptor Gamma Gene Rearrangement Assay 2.0 targets more regions compared to the ***TCRG*** Gene Clonality Assay, it detects more types of rearrangements. Furthermore, the different primer designs used by these assays could exhibit variability when comparing results generated by the two assays. If a primer binding site utilized by the T-Cell Receptor Gamma Gene Rearrangement Assay 2.0 is mutated, the intended primer may not bind efficiently and result in low amplification of the target in question; ***TCRG*** Gene Clonality Assay primers may bind at a different location and amplify the same target. The opposite may be true as well.

Overall, we do expect a high degree of concordance between the two assays, but it is not expected to be 100%, as these are different assays with unique primer designs. The following references compare the and describe the characteristics and workflow parameters of the one-tube and/or two-tube assays:

Assay Design Affects the Interpretation of T-Cell Receptor Gamma Gene Rearrangements[1]
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2963914/

A Single-Tube, EuroClonality-Inspired, *TRG* Clonality Multiplex PCR Aids Management of Patients with Enteropathic Diseases, including from Formaldehyde-Fixed, Paraffin-Embedded Tissues[2]
https://jmd.amjpathol.org/article/S1525-1578(18)30173-9/pdf

A New and Simple *TRG* Multiplex PCR Assay for Assessment of T-cell Clonality[3]
https://journals.lww.com/hemasphere/Fulltext/2019/06000/A_New_and_Simple_TRG_Multiplex_PCR_Assay_for.8.aspx

3. Additional Positive Controls

While both assay kits include positive and negative controls, Invivoscribe offers additional controls that are compatible with these kits. Please see Table 1 for details.

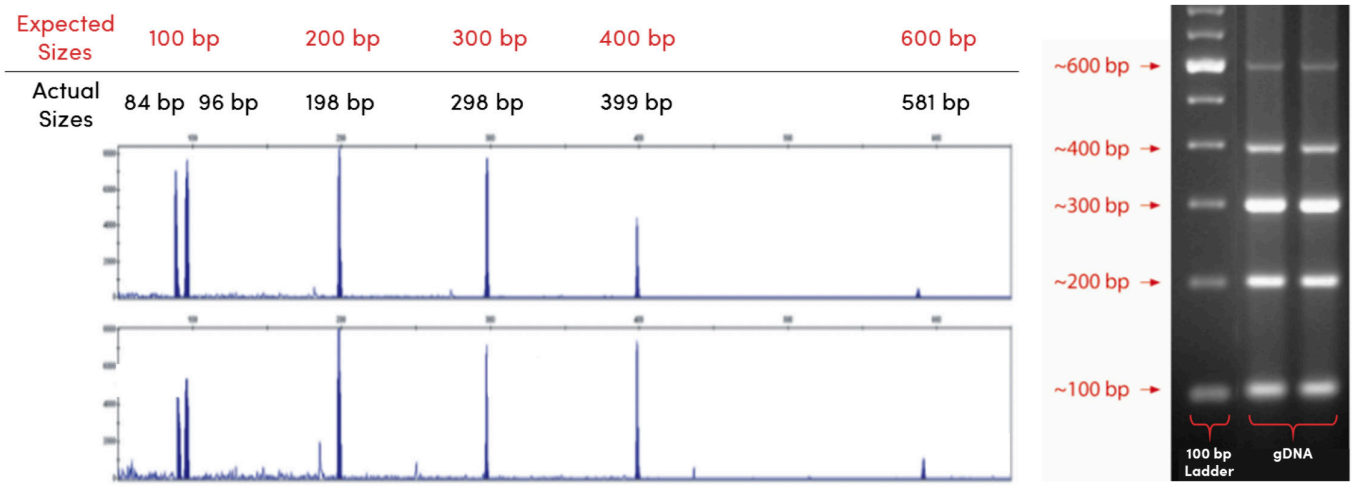
Table 1. Positive Controls Compatible with either or both *TCRG* assays

Part Number	Description	Product Size(s)	
		***TCRG* Gene Clonality Assay**	**T-Cell Receptor Gamma Gene Rearrangement Assay 2.0**
40880190	100% IVS-0004 Clonal Control DNA	***TCRG*** Tube A: 211 bp ***TCRG*** Tube B: n/a	***TCRG*** – 6FAM: 179 bp and 188 bp
40880430	100% IVS-0008 Clonal Control DNA	***TCRG*** Tube A: 218 bp and 229 bp ***TCRG*** Tube B: n/a	***TCRG*** – 6FAM: 195 bp and 207 bp
40880490	100% IVS-0009 Clonal Control DNA	***TCRG*** Tube A: 212 bp ***TCRG*** Tube B: 115 bp	***TCRG*** – 6FAM: 188 bp and 191 bp
40881210	100% IVS-0021 Clonal Control DNA	***TCRG*** Tube A: 211 bp ***TCRG*** Tube B: 143 bp and 167 bp	***TCRG*** – 6FAM: 183 bp and 188 bp

4. Sample Integrity

Invivoscribe capillary fluorescence assays generate robust results using genomic DNA (gDNA) extracted from a wide array of specimen types. If the integrity of the sample is in question, the Specimen Control Size Ladder master mix can be used to verify the quality and quantity of the extracted gDNA is suitable to generate results with Invivoscribe assays. Specimen Control Size Ladder master mixes are included with most Invivoscribe kits, including the T-Cell Receptor Gamma Gene Rearrangement Assay 2.0. Examples of the expected results for Gel and ABI Fluorescence detection with this master mix are shown in Figure 2, respectively:

Figure 2: Expected results amplifying gDNA with: (Right) the Specimen Control Size Ladder - Unlabeled for Gel Detection (Catalog #20960020). Expected product size are indicated in **red**; a 100 bp ladder is also shown for size reference. (**Below**) the Specimen Control Size Ladder – 6FAM for ABI Fluorescence Detection (Catalog #20960021). Expected product sizes are indicated in **red** and actual product sizes are indicated in **black**.



5. Sample Interpretation

Many publications have indicated T-cell receptor gamma gene rearrangement analysis is useful as an ancillary test in the diagnosis of T-cell neoplasms.[1-6] However, the design of the assay can have a large impact on assay interpretation, correlating increasing assay complexity with more complicated and difficult interpretation.[1,3]

The T-Cell Receptor Gamma Gene Rearrangement Assay 2.0 was designed with this consideration in mind, simplifying interpretation by using an individual, yet comprehensive, master mix labeled with one fluorochrome that generates a single, robust valid size range. This is a major improvement in comparison to the ***TCRG*** Gene Clonality Assay, which uses two master mixes labeled with two fluorochromes that generate a total of eight valid size ranges for interpretation. A comparison of assay interpretation guidelines is provided in Table 2.

The higher complexity of the ***TCRG*** Gene Clonality Assay can lead to false positive results due to low-amplitude polyclonal distributions with pseudoclonal peaks.[1] Because of the limited repertoire generated by ***TRG*** rearrangements, amplification of a sample with a high tumor load and limited number of T-cells can generate products that appear to be (oligo)clonal or pseudoclonal.[6]

Table 2. Comparison of Invivoscribe *TCRG* Assay Interpretation Guidelines

Interpretation by Assay		Final Call (Sample Report)
TCRG **Gene Clonality Assay** **Valid Size Range** ***TCRG*** Tube A: 145 bp – 255 bp ***TCRG*** Tube B: 80 bp – 220 bp*	**T-Cell Receptor Gamma Gene Rearrangement Assay 2.0** **Valid Size Range** ***TCRG*** – 6FAM: 159 bp – 207 bp	
1 or 2 significant peaks within the valid size range(s). **Significant Peak:** 3X amplitude of 3rd highest peak within the same polyclonal distribution	1 or 2 significant peaks within the valid size range **Significant Peak:** 3X amplitude of lower neighboring peak	Detection of ***TCRG*** Gene Rearrangements, consistent with the presence of a clonal cell population.
N/A	3 or more significant peaks within the valid size range **Significant Peak:** 3X amplitude of lower neighboring peak	Detection of ***TCRG*** Gene Rearrangements, consistent with the detection of biclonality or oligoclonality.
Absence of significant peaks within the valid size range(s)	Absence of significant peaks within the valid size range	Negative for detection of ***TCRG*** Gene Rearrangements.

*Interpret peaks within context of all sample information.

6. Protocol Tips: T-Cell Receptor Gamma Gene Rearrangement Assay 2.0

Importing Plate Layout into GeneMapper

1. Open a new project and select **Microsatellite** for the project type.
2. Add raw data files derived from CE analysis to the project using the ***Add samples to Project*** icon in the toolbar.
3. Once the samples are added, verify that ***Analysis Method*** selected is ***Microsatellite Default*** and that the appropriate ***Size Standard*** is selected.

    - If necessary, select samples and press the **Analyze** green play button in the toolbar.
    - There will be a **green**, **yellow**, or **red** symbol in the SQ column for each analyzed sample.

4. If the symbol is **red**, the software will not calculate the peak sizes or display the sample data. In this case, view the size standard chart by selecting samples and clicking the **Size Match Editor** icon in the toolbar to verify the peak sizes were correctly identified.

    - If the SQ peaks were called correctly, click **Override SQ**, and then **Apply**.
    - If some peaks were called incorrectly, re-assign the peaks by right clicking and selecting the correct size values.

        - The GS 400 HD ROX size standard provides 21 labeled fragments of: 50, 60, 90, 100, 120, 150, 160, 180, 190, 200, 220, 240, 260, 280, 290, 300, 320, 340, 360, 380, and 400 nucleotides (nt).
        - The GS 600 LIZ Size Standard provides 36 single-stranded labeled fragments of: 20, 40, 60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400, 414, 420, 440, 460, 480, 500, 514, 520, 540, 560, 580 and 600 nt.

5. Select any samples that have a green play button in the ***Status*** column of the GeneMapper table and press the **Analyze** green play button in the toolbar.

    - Please note, it may be necessary to lower the Minimum Peak Height threshold in order to detect all peaks in a Gaussian distribution:

        - Select **GeneMapper Manager** from the ***Tools*** menu, navigate to the ***Analysis Methods*** tab, and open the ***Microsatellite Default Analysis Method Editor***.
        - Navigate to the ***Peak Detector*** tab, select **User Specified (rfu)** toggle, and input the desired peak height for 6FAM dye.

    - If changes are made, click the **Analyze** green play button in the toolbar.

6. In the GeneMapper table, highlight the samples of interest and select **Display Plots** in the GeneMapper toolbar.
7. Once the Display Plot is open, verify that ***Microsatellite Default*** is selected in ***Plot Settings*** and the appropriate fluorophore is selected (6FAM).

    - Select/Deselect the associated colored boxes in the toolbar, OR navigate to the ***View*** menu in the ***Display Plot***, select **Dyes**, and select the appropriate dyes.

        - As a reminder, 6FAM is **Blue**, HEX is **Green**, NED is **Yellow** or Black and ROX is **Red** in GeneMapper v4.0.

8. In order to view the peak size and height, select the **Sizing Table** icon in the toolbar.

Recommended Injection Parameters and Peak Detector Settings for the T-Cell Receptor Gamma Gene Rearrangement Assay 2.0

Injection Parameters*

Parameter	Value
Injection Time	7 sec.
Injection Voltage	1.0 kVolts
Capillary Length	50 cm
Polymer	POP7
Dye Set	G5
Oven Temp	60°C
Run Time	1630 sec.
Run Voltage	19.5 kVolts
PreRun Time	180 sec.
PreRun Voltage	15 kVolts
Data Delay	1 sec.

Peak Detector Settings*

Parameter	Value
Peak Detector Algorithm	Basic
Minimum Peak Height	User Specified (RFU)
Blue	50
Orange	50
Valid Size Range	159 bp - 207 bp

*Please refer to the user manual for further instructions.

Analyzing Samples in GeneMapper

- Examine all assay controls prior to interpretation of sample results. If the controls do not yield the correct results, the assay is not valid and the samples should not be interpreted.
- When analyzing the samples in GeneMapper, only analyze peaks within the valid size range of the master mix.
- To determine whether a peak is clonal or polyclonal, compare the height of the peak to the height of the polyclonal background within the same sample.
    - o A validation study is recommended to determine an appropriate peak height ratio.
- Do not use RFU values to determine whether a peak is clonal, as RFU values can be affected by a variety of factors, such as the capillary array and laser life.

Troubleshooting Size Quality (SQ) Error(s)

A SQ Error indicates that GeneMapper did not identify the size standard peaks correctly, causing the generated products to be identified at an incorrect size. Most SQ errors are caused by saturated signal(s), resulting in spectral pullup and mis-identification of peak sizes. If this type of error is observed in undiluted positive controls and the size standard peaks are identified correctly or corrected, the SQ Error is of no significance since control validity relies on the presence or absence of a peak at the expected size. However, if signal saturation is observed in the negative control, sensitivity controls, or unknown samples, the run must be repeated on the ABI instrument to prevent result misinterpretation. Before repeating the ABI run with samples exhibiting saturated signal(s), adjust the injection conditions and/or dilute amplified products. Please note that diluting unknown sample amplicons could dilute a weak clonal product below the assay level of detection. The steps below will correct SQ Errors:

1. Verify the size standard peaks are correctly identified in the GeneMapper software prior to reviewing the data by selecting the samples then clicking the **Size Match Editor** icon in the tool bar.
2. If the peaks labeled with SQ Errors were called correctly, click **Override SQ**, then click **Apply**.
3. If the size standard peaks are identified incorrectly, (in the ***Size Match Editor***) manually reassign the ROX size standard peaks to match the expected sizes: Right click on the incorrect size value and select the correct size value, then click the **Override SQ** button.
4. Repeat step(s) 2 or 3, above, for all samples indicating an SQ Error based on the peak size verification result (step 1).
5. Click the **Analyze** green play button to reanalyze the data.
    - The data will update to display the revised product sizes.

If SQ Errors continue to occur, recalibrate the ABI instrument per the manufacturer instructions. Further optimization to reduce the number of SQ Errors includes adjusting the volume of size standard added to each well of the ABI plate.

Troubleshooting Miscalled/Unidentified Peaks

Another potential issue that may be encountered includes unidentified peaks which are miscalled as belonging to an adjacent peak. This phenomena occurs when the electropherogram indicates two adjacent peaks and the peak table only indicates one peak. Subsequently, when this data is pasted into the Algorithm Worksheet, the calculation does not identify a potentially significant peak because there is no adjacent peak to which it can be compared. This can easily be remedied by changing the Peak Detection settings in GeneMapper, as the software uses the settings ***Polynomial Degree*** and ***Peak Window Size*** to determine the start and end of each peak. The default value for Polynomial Degree is 3 and Peak Window Size is 15. Please see Thermo Fisher language regarding these settings below:[7]

- Polynomial Degree and Peak Window Size settings affect the peak detection sensitivity. Adjust these parameters to detect a single base pair difference while minimizing the detection of shoulder effects and/or noise (see Table 3 and Figure 3)

Table 3. Change peak detection sensitivity using Polynomial Degree and Peak Window Size

Function	Polynomial Degree Value	Peak Window SizeValue
Increase Sensitivity	Higher	Lower
Decrease Sensitivity	Lower	Higher

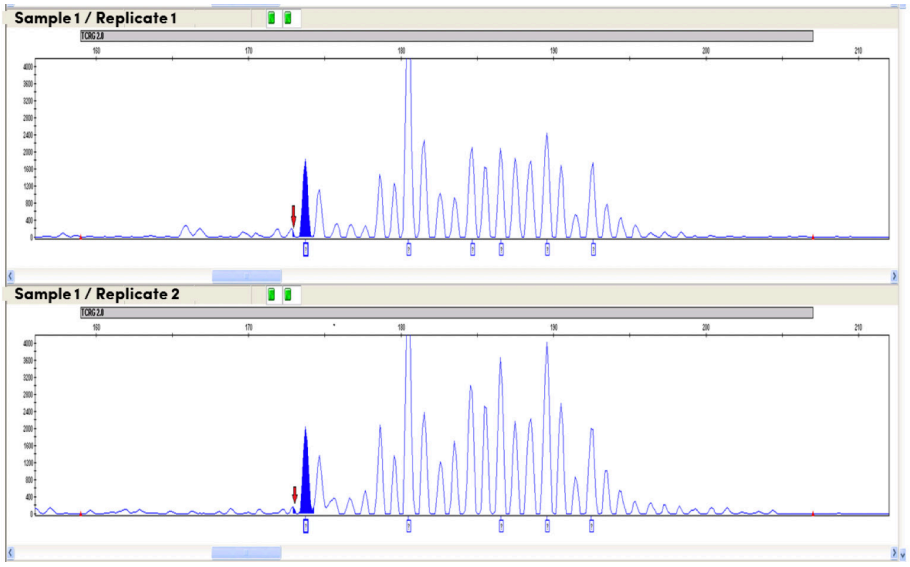
- The peak window size functions with the polynomial degree to set the sensitivity of peak detection. The peak detector calculates the first derivative of a polynomial curve fitted to the data within a window that is centered on each data point in the analysis range.
- Using curves with larger polynomial degree values allows the curve to more closely approximate the signal and, therefore, the peak detector captures more of the peak structure in the electropherogram.
- The peak window size sets the width (in data points) of the window to which the polynomial curve is fitted to data:
    - o Higher peak window size values smooth out the polynomial curve, which limits the structure being detected.
    - o Smaller window size values capture more of the peak structure.

To adjust these settings, follow the steps below:

1. Open ***GeneMapper*** and from the top menu, select **Tools**, then **GeneMapper Manager**.
2. Navigate to the ***Analysis Methods*** tab to view available analysis methods.
    - An existing ***Analysis Method*** can be edited, or (the preferred option) an existing ***Analysis Method*** can be edited then saved with a new name.
3. Select **Save as...** to create a copy of the selected analysis method with a new name.
4. Select the ***Analysis Method*** created in the previous step and click **Open**.
5. Navigate to the ***Peak Detector*** tab, and set ***Peak Detection Algorithm*** to **Advanced**.
    - The ***Peak Window Size*** and ***Polynomial Degree*** settings can now be altered.
6. Test a few different conditions with a sample set to identify the optimal settings for this instrument.
    - Use the guidelines in Table 3 to determine how to adjust these settings based on the observed issue and anticipated resolution.

Figure 3. Sample data generated with GeneMapper using the default *Peak Detection Algorithm* settings vs. optimized *Peak Detection Algorithm* settings.

Default ***Peak Detection Algorithm*** settings: ***Polynomial Degree*** = 3; ***Peak Window Size*** = 15. Notice the peak indicated by the red arrow is not identified in the peak table, rendering the 173 bp peak as not significant (since it is compared to the higher peak on the left).



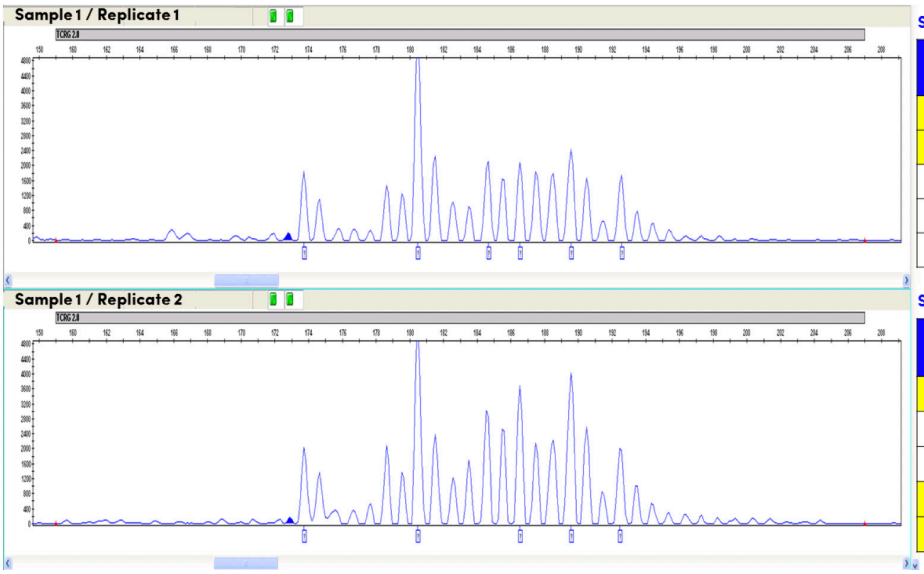
Summary Table: Sample 1/Replicate 1

Peak #	Product size	Peak height	RPR	Significant?
1	180.51	7203	**5.91**	Yes
2	192.54	1686	2.87	No
3	189.63	2407	1.50	No
4	184.78	2021	2.05	No
5	174.67	1087	2.85	No

Summary Table: Sample 1/Replicate 2

Peak #	Product size	Peak height	RPR	Significant?
1	180.48	6919	5.10	Yes
2	189.63	3879	1.74	No
3	186.58	3587	1.64	No
4	192.48	2067	2.53	No
5	178.74	2002	3.58	No

Optimized ***Peak Detection Algorithm*** settings: ***Polynomial Degree*** = 3; ***Peak Window Size*** = 11. Notice the peak indicated by the red arrow is now identified in the peak table, rendering the 173 bp peak as **significant** (since it is now compared to the lower peak on the right).



Summary Table: Sample 1/Replicate 1

Peak #	Product size	Peak height	RPR	Significant?
1	180.48	5846	4.78	Yes
2	173.82	1854	7.82	Yes
3	192.57	1703	3.17	No
4	184.62	2109	2.12	No
5	189.71	2489	1.51	No

Summary Table: Sample 1/Replicate 2

Peak #	Product size	Peak height	RPR	Significant?
1	180.54	5794	4.16	Yes
2	189.71	4306	1.95	No
3	186.52	3712	1.70	No
4	173.79	2044	9.42	Yes
5	178.79	2103	4.31	Yes

Available Products

Catalog Number	Description	Quantity
12070101	T-Cell Receptor Gamma Gene Rearrangement Assay 2.0	33 reactions
12070111	T-cell Receptor Gamma Gene Rearrangement Assay 2.0 MegaKit	330 reactions

These products are for Research Use Only. Not for use in diagnostic procedures.

References

1. Cushman-Vokoun, AM ***et al.,*** (2010) ***Journal of Molecular Diagnostics*** 12(6):787-796. doi: 10.2353/jmoldx.2010.090183

2. Derrieux, C ***et al.,*** (2019) ***Journal of Molecular Diagnostics*** 21(1): 111-122. doi: https://doi.org/10.1016/j.jmoldx.2018.08.006

3. Armand, M ***et al.,*** (2019) ***Hemasphere*** 3(3): e255. doi: 10.1097/HS9.0000000000000255

4. Langerak, A ***et al.,*** (2012) ***Leukemia*** 26, 2159–2171. doi: https://doi.org/10.1038/leu.2012.246.

5. van Krieken, J ***et al.,*** (2007) ***Leukemia*** 21, 201–206. doi: https://doi.org/10.1038/sj.leu.2404467

6. van Dongen, J ***et al.,*** (2003) ***Leukemia*** 17, 2257–2317. doi: https://doi.org/10.1038/sj.leu.2403202

7. Applied Biosystems 3500/3500xL Genetic Analyzer User Guide (Part Number 4401661 Version: c). https://tools.thermofisher.com/content/sfs/manuals/4401661.pdf

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