Ewing’s Sarcoma RT-PCR Assays

**Principle and Clinical Indications:**

About 86% of Ewing’s sarcoma (EWS) contain a fusion of the EWS and FLI1 gene, due to the t(11;22)(q24;q12) translocation. At the molecular level, the EWS-FLI1 rearrangement show many different combinations of exons from EWS and FLI1. These combinations encode in-frame fusion transcripts and results in differences in the length of the chimeric protein. Among the t(11;22) translocation, the most common type is type 1 and the second common is type 2 can be detected by RT-PCR assay. A smaller subset of EWS cases (13%) expressed a variant fusion protein generated by t(21;22) translocation involving ERG and EWS gene which can also be detected with RT-PCR method. This assay may be indicated whenever a diagnosis of Ewing’s sarcoma is under consideration, or as mandated by clinical protocol. The primers and probes used in this assay are published in Lewis et al, “Differentiating Ewing’s sarcoma from other blue cell tumors using a RT-PCR translocation panel on formalin-fixed paraffin-embedded tissues”. (Modern Pathology, (2007), 20, 397-404).

**A) REAGENTS**

- QuantiTect Multiplex PCR Kit (Qiagen cat# 204541)
- SYBR Green PCR Master Mix (ABI cat# 4309155)
- 96 well plates (Applied Biosystems)
- Primers/Probes (Applied Biosystems)

**Primers:**

- EWS-E7-FW: cca agt caa tat agc caa cag
- FLI1-E6-Rev: ggc cag aat tca tgt tat tgc
- ERG-REV : tcc agg agg aac tgc caa ag
- B2M-F: tga ctt tgt cac agc cca aga ta
- B2M-R: aaa tgc ggc atc ttc aaa cc
Probe: ABI –MGB type :
  EWS-FLI1 for EWS Type 1
    FAM - acg-ggc-agc-aga/acc-ctt-ctt-at - MGBNFQ
  EWS-FLI1 for EWS Type 2
    FAM – acg-ggc-agc-aga/gtt-cac-tgc-t - MGBNFQ
Beta2Microglobulin probe for house keeping gene:
    VIC- tcg aga cat gta agc agc at- MGBNFQ

Primer set and probe are pre-mixed and stored in multiple aliquots in -20C as follow:
  1) B2M mix (20x): Primer F/R 6uM each, VIC probe 2uM. Amplicon size 88bp.

  2) EWS type 1 mix (20x): Primer EWS-E7-FW / FLI1-E6-Rev 8uM each,
      Type 1 FAM probe 4uM. Amplicon size 100bp.

  3) EWS type 2 mix (20x): Primer EWS-E7-FW / FLI1-E6-Rev 8uM each,
      Type 2 FAM probe 4uM. Amplicon size 166bp

  4) EWS ERG mix (25x): Primer EWS-E7-FW / ERG-REV 5uM each.
      Amplicon size 154bp.

B) Procedure:
  1) cDNA Synthesis: please follow SOP – cDNA Synthesis. A negative RNA control (HELA cell line RNA) and positive cell line RNAs (EWS type 1 - TC71; EWS type 2 – CHPL; EWS ERG – 67R) will need to be included in this procedure.

  2) ViiA 7 PCR set up:

Before setting up, turn on ViiA7 instrument and operating PC.
PCR CONDITION :
Stage 1: 1 cycle: 95°C 15 min  
Stage 2: 40 cycles: 95°C 45 sec  
60°C 60 sec  

Volume per well: 25uL  

PCR cycling and condition is pre-programmed in the template “EWS-ARMS-SYN”. SYBR-Green assay requires melting step at the end if patient sample is positive for amplification. The condition is pre-programmed in EWS-ERG template.

3) Prepare 3 master mixes: EWS-type 1, EWS-type 2 and EWS-ERG

**EWS-Type1 Master Mix reaction (DUPLEX PCR)**

<table>
<thead>
<tr>
<th></th>
<th>1 reaction (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QuantiTect Multiplex Buffer 2X</td>
<td>12.5</td>
</tr>
<tr>
<td>Type 1 primer/probe mix</td>
<td>1.25</td>
</tr>
<tr>
<td>B2M mix</td>
<td>1.25</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>7.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>22.5</strong></td>
</tr>
</tbody>
</table>

**EWS-Type2 Master Mix reaction:**

<table>
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<tr>
<td>QuantiTect Multiplex Buffer 2X</td>
<td>12.5</td>
</tr>
<tr>
<td>Type 2 primer/probe mix</td>
<td>1.25</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>8.75</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>22.5</strong></td>
</tr>
</tbody>
</table>

**EWS-ERG Master Mix reaction: SYBR-Green PCR**

<table>
<thead>
<tr>
<th></th>
<th>1 reaction (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR Green Master Mix 2X</td>
<td>12.5</td>
</tr>
<tr>
<td>EWS-ERG primer mix</td>
<td>1.0</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>9.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>22.5</strong></td>
</tr>
</tbody>
</table>
Sample cDNA input: 2.5 μL, all assays are run in duplicate.

- Add 22.5 μL of your complete Master Mix.
- Aliquot 2.5μL of positive control, negative control, NTC, and unknown samples into the appropriate wells.
- Cover with the clear plastic cover and seal the wells tightly.
- Spin down the 96-well plate for 2 min to rid of bubbles in the wells.
- Enter the samples into worksheet, save as date of the run and assay name (mmddyr-EWS format) in “CLINICAL RUNS” folder.
- Place into the plate holder of the V7A7 machine.
- Click green icon to start the run.

C) Data Analysis and Report Generation:
- When the run is complete, there will be a message from the software.
- Click on “Analyze” icon, the data will be analyzed and the amplification curves may be visualized.
- Setup threshold as 0.005 Ct, Baseline starts ct=6, ends at ct=18
- Print data and sample amplification plot.

D) Quality Control:

Every 96-well-plate RT-PCR run includes positive control, negative control, and no-template control (NTC). All control materials much be handled identically as the samples. The test result is valid when all following criteria meet: 1) positive controls fall in range and Cts of negative control and NTC are ≥ 40 cycles. 2) B2M internal control is ≤ 35 cycles when sample is negative. If non-valid results occur, repeat the test.

E) Interpretation of Test Results

Positive and Negative Results: positive or negative test result will be
reported for all samples. The cut-off value was established base on initial validation.

- Positive: EWS-Type1 or EWS-Type2 or EWS-ERG Ct < 38 with adequate Melting Curve.
- Negative: Ct ≥ 38 cycles AND B2M Ct ≤ 35 (indicates RNA is adequate to provide required sensitivity).
- No Molecular Diagnosis Rendered: EWS-Type1 and EWS-Type2 and EWS-ERG Ct ≥ 38 cycles AND B2M Ct > 35 (indicates that RNA is inadequate to provide required sensitivity).

Recorded: 02/21/2007
Revised: 02/10/2009 by Liqiang Xi, MD.
Revised: 12/04/2013 by Liqiang Xi, MD.
Reviewed and Approved: 12/04/2013 by Mark Raffeld, M.D.
Revised: 02/02/2016 by Tina Pham, MT. and Liqiang Xi, MD.