Technology seminar:

Validation of NGS and RNA-sequencing data by qPCR technologies

Dr. Sunny Wong, Roche Diagnostics
Basic application: Quantification

**Absolute Quantification**

**Typical Application Fields:**
- Identification of a specific species (e.g. bacteria, virus)
- Detection of specific DNA/RNA (e.g. oncology research)
- Pathogen detection (e.g. legionella, anthrax)
- Antibiotic resistance screening (e.g. MRSA, VRE)
- Water quality monitoring
- ...

**Relative Quantification**

**Typical Application Fields:**
- Determination of mRNA expression levels (e.g. cytokines, chemokines)
- Gene dosage quantification (e.g. chromosomal aberrations)
- Studies on minimal residual diseases (MRD)
- GMO detection
- ...

![Diagram showing the process of quantification](image)
Basic application: Endpoint Genotyping

- Two sequence-specific hydrolysis probes, one designed for wildtype, one for mutant target DNA, and labeled with different dyes.

- Genotype determination by measuring the intensity distribution of the two dyes after PCR.
Advanced qPCR technologies for NGS data validation

• Melt curve based method
  - LightSNiP assay for validating SNP
    - High resolution melting for validating SNP and complicated variants
• Universal ProbeLibrary
  - benefits your workflow in RNA-Seq validation
Advanced application: Melting Curve Based Method

HybProbe

Denaturation

Annealing

Elongation

SimpleProbe

R = Reporter (Fluorescein)
L = Linker

5’-p 3’

5’-p 3’
Advanced application: Melting Curve Based Method

*HybProbe*

<table>
<thead>
<tr>
<th>Low</th>
<th>Medium</th>
<th>High</th>
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</thead>
</table>

- **Mismatch**
- **Perfect Match**

**Temperature**

- Low
- Medium
- High
Melt curve based method
Validation of SNP

Melting Curves
Fluorescence

Melting Peaks
-dF/dT

*Mismatch
Perfect Match

*Mismatch
Perfect Match

°C Temperature
Melt curve based method
Validation of SNP with Three Different Alleles

Template: Plasmid DNAs
Target: Apolipoprotein B
Single Color: LC RED 640
**LightSNiP**

**rs6921948 HCG27**

Preparation of parameter-specific reagents (96 reactions):

One reagent vial contains all primers and probes to run 96 LightCycler® reactions. Spin vial before opening to ensure the yellow pellet is located at the base of the reaction tube. Add 100 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.

**Melting Curves**

**Melting Peaks**
Advanced qPCR technologies for NGS data validation

• **Melt curve based method**
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• **Universal ProbeLibrary**
  - benefits your workflow in RNA-Seq validation
Amplicon Melting

Principle of Gene Scanning by HRM

- DNA with heterocrygote SNP
- PCR
- Homoduplexes
- Denaturation, reannealing, intercalating fluorescent dye
- Increasing temperature
- Heteroduplexes

www.roche-applied-science.com
Gene Scanning
High-Resolution Melting Analysis for SNP validation

Example:
Sequence variations (SNP G→T) in the LPLH3 gene
72 samples, 164 bp amplicon
Rapid identification of multidrug-resistant Mycobacterium tuberculosis isolates by rpoB gene scanning using high-resolution melting curve PCR analysis.

Ariane T. Pietzka, Alexander Indra, Anna Stoger, Josef Zeinzinger, Miriam Konrad, Petra Hasenberger, Franz Allerberger and Werner Ruppitsch


This publication concluded that, with a positive predictive value of 100% and a negative predictive value of at least 99.9%, this combined HRM curve analysis is an ideal screening method for the TB laboratory, with minimal requirements of cost and time. The method is a closed-tube assay that can be performed in an interchangeable 96- or 384-well microplate format enabling a rapid, reliable, simple and cost-effective handling of even large sample numbers.

(http://jac.oxfordjournals.org/content/63/6/1121.full)
High-Resolution Melting
An useful tool for validating NGS data

Comparison of high resolution melting analysis, pyrosequencing, next generation sequencing and immunohistochemistry to conventional Sanger sequencing for the detection of p. V600E and non-p.V600E BRAF mutations

Michaela Angelika Ihle, Jana Fassunke, Katharina Konig, Inga Grunewald, Max Schlaak, Nicole Kreuzberg, Lothar Tietze, Hans-Ulrich Schildhaus, Reinhard Buttner and Sabine Merkelbach-Bruse

“The assay was set up with an amplicon of 163 base pairs and is therefore able to detect all hotspot mutations as well as rare mutations in the entire exon 15 (codon 582 to 620) of BRAF (specificity 100%).”

- As sensitive as Sanger sequencing
- Achieved 100% specificity
- Rapid and cost effective, close-tubed
Evaluation of High-Resolution Melting Analysis as a Diagnostic Tool to Detect the BRAF V600E Mutation in Colorectal Tumors

Martin Pichler,* Marija Balic,∗ Elke Stadelmeyer,∗ Christoph Ausch,† Martina Wild,‡ Christian Guelly,§ Thomas Bauernhofer,* Hellmut Samonigg,* Gerald Hoefler,‡ and Nadia Dandachi*

From the Division of Oncology, * Department of Internal Medicine, Medical University of Graz, Graz, Austria; the Department of Surgery, † Ludwig Boltzmann Research Institute of Surgical Oncology, Danube Hospital, Vienna, Austria; the Institute of Pathology, ‡ Medical University of Graz, Graz, Austria; and the Center for Medical Research, § Medical University of Graz, Graz, Austria

Journal of Molecular Diagnostics, Vol. 11, No. 2, March 2009

“In the comparison of HRM with DNA sequencing and DHPLC, respectively, neither sequencing nor DHPLC were able to detect 5% mutated cell DNA. The advantage of HRM to detect even 1% of cell line DNA known to harbor BRAF V600E may be of importance when only tissues with a low proportion of tumor cells are available.”
High-Resolution Melting

Novel Method With Many Potential Applications

The main application for the HRM method is gene scanning \textit{i.e.}, the discovery of new variants in target gene-derived PCR amplicons.

Possible applications of High Resolution Melting:
• Genotyping of known SNPs
• Characterization of haplotype blocks
• Screening for loss of heterozygosity
• Allelic prevalence in a population
• Species identification/taxonomy
• DNA mapping (find individuals with many highly variable, informative loci)
• Identification of candidate predisposition genes
• DNA methylation analysis
• ....
**LightCycler® 480 Real-Time PCR System**

**Technical Note No. 1**

**High Resolution Melting: Optimization Strategies**

High resolution melting (HRM) is a novel, closed-tube, post-PCR technique allowing genomic researchers to easily analyze genetic variations in PCR amplicons.

This technical note describes general steps of setting up HRM-based PCR assays, with a special focus on ways to optimize procedures for gene scanning experiments.
**LightCycler® 480 Instrument Heat Sink**

*Therma-base*

- Thin sealed vacuum vessel with working fluid in a wick structure
- Rapidly transfers heat by evaporation and condensation

→ enables both rapid and accurate cycling!
Thermal uniformity
Instrument comparison

LightCycler® 480 Instrument  Standard Instrument
LightCycler® 480 Optical System

Minimized edge effect

- **White LED**
  - high intensity
  - broad dynamic range
  - lifetime
  - for LED: approx. 10,000 hrs

- **CCD camera**

- **Five excitation filters**

- **Six detection filters**

- **Optimized arrangement of optical components**

- **Homogeneous excitation and fluorescence detection**
Gene Scanning Experiment

Data Analysis

- Amplification
- High Resolution Melting
  - Normalization
  - Temperature Shift
  - Difference Plot

[Graphs and diagrams showing amplified results and melting curves]

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Validation of RNA-Seq data with qPCR

**TaqMan**
- Gold standard
- Specific and sensitive
- Commercial available
- Expensive
- Long delivery time

**SYBR Green**
- Flexible
- Low Cost
- Suitable for screening many gens
- Non-specific
- Need optimization

Can I have the advantages of both products?
Universal ProbeLibrary (UPL) — facilitate customers to design custom gene expression assays to quantify virtually any transcript in any genome.
Universal ProbeLibrary (UPL)

How does it work? – Universal

gtacactgacacccaggttgcctccctctgacttcagtaagcagacaccactccactctctccactttgacgctgggctggcattgccctcaacgaccactttgtcaagctaatga
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Universal ProbeLibrary (UPL)

How does it work? – Specificity
Universal ProbeLibrary (UPL)
Fast delivery time of probes

Benefit of Fast Workflow

**Universal ProbeLibrary Workflow**

**DAY 1**
- Identify gene or target sequence of interest
- Design custom assay at [www.universalprobelibrary.com](http://www.universalprobelibrary.com)
- Order assay-specific primers from your preferred oligo supplier for overnight delivery

**DAY 2**
- Select the appropriate probe from the Universal ProbeLibrary Set in your freezer
- Perform real-time PCR
- Evaluate results
Assay design in 3 simple steps

www.universalprobelibrary.com

Universal ProbeLibrary Assay Design Center

Design RT-qPCR assays, including the probe and gene specific primer for your targets of choice, in just 3 easy steps.

Simply:
1. Select your organism
2. Enter your gene accession number, gene name, or sequence
3. Click Design

   1. Start by selecting your target organism below:
      Select organism

Explore the links below for more information, including how to combine target assays with Universal ProbeLibrary reference gene assays.

Reference Guides

Promotions

Special Offers

Cell Lysis
A simple, one-step solution. Request a sample.
> Learn more!

Universal ProbeLibrary
Easy assay design today; qPCR results tomorrow
> Learn more!

What type of researcher are you?
Take the quiz to find out.
> Learn more!
> All Promotions
Assay design in 3 simple steps

1. Choose Your Organism

Universal ProbeLibrary Assay Design Center
Roche Applied Science

The ProbeFinder software allows you to perform a fast and easy design of real-time PCR assays for your targets of choice. It will select an optimal combination of a Universal ProbeLibrary probe and a gene-specific primer set.

To start the design process, select an organism:
- enter the gene accession number, gene name, or paste a sequence in the appropriate field.

Assays for Human, Mouse or Rat targets can now be combined with a reference gene assay.

Need more information?
- Design batch assays for up to 10 independent assays at a time
- Find common assays for all members of a gene family
- Discriminate them as well as splice variants of a gene.

The ProbeFinder Quick reference gives you a short guidance on how.

For a detailed description of all features of ProbeFinder, you can download the guide.
Assay design in 3 simple steps
Assay design in 3 simple steps

1. Choose Your Organism
2. Type in Your Gene of Interest
3. Hit Design
Get custom assay design in 60 seconds

Supporting MIQE Compliance

Use Universal ProbeLibrary probe: #64, cat.no. 04688635001

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Amplicon (121 nt)

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Primer sequences

Amplicon sequence/length

Exon junction info

Probe sequence
Universal ProbeLibrary (UPL)
Applications – Gene expression

• **5 Reference Gene Assays** are available for multiplexing:
  – 3 human (ACTB, GAPD, G6PD)
  – 2 mouse (m-ACTB, m-GAPD)

• Second dye (**LightCycler ® Yellow 555**) detectable in the VIC/HEX channel.
**Universal ProbeLibrary (UPL)**

*Other applications*

- Gene Expression
- Genotyping
- miRNA Quantification
- Copy Number Variation
Roche provides solution for a broad range of applications

- **LightCycler480**
  - Reagent
  - Assay

- **Genotyping**
  - Endpoint
  - Melt curve
  - High resolution melting

- **Quantification**
  - Methylation
  - Methylation Quantification

- **Complicated mutations**

- **Haplotyping**

- **Low variant mutation**

**Rapid and accurate ways to validate challenging NGS data**
Promotional Offer
Until 31 Dec 2016

• **First Trial 50% Discount on 5ml pack**
  04707516001 LC480 SYBR Green I Master 5x1ml
  04707494001 LC480 Probes Master 5x1ml

• **Bulk Pack Discount (Buy 2 get 1 free on 50ml pack)**
  04887301001 LC480 SYBR Green I Master 10x5ml
  04887352001 LC480 Probes Master 10x5ml

For enquiries, please contact:

**Mavis Yeung** at [mavis.yeung@roche.com](mailto:mavis.yeung@roche.com) or 97662601
Doing now what patients need next