Testing (DRAFT)

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Testing Platforms

Three Platforms:

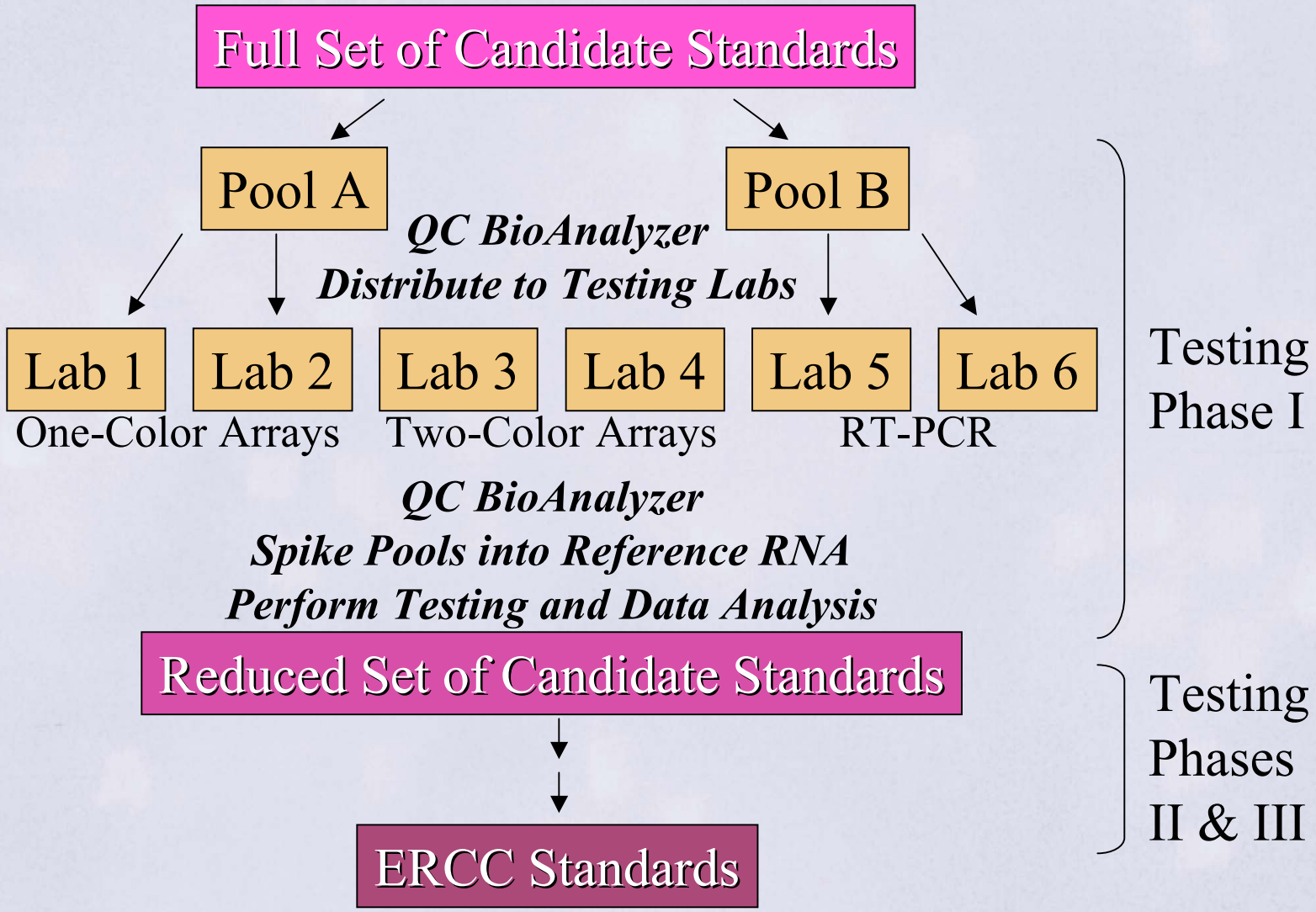
1. One-Color Microarrays
   One biotin-labeled target per hybridization
   Multiple probes per transcript (e.g. Affymetrix)
2. Two-Color Microarrays
   Two fluorescent-labeled (Cy3 & Cy5) targets per hybridization
   Single probe per transcript (e.g. Agilent)
3. Quantitative RT-PCR
   SYBR green, single tube procedure

Amersham? ABI? Spotted?
Alternative RT-PCR Methods?

Testing Considerations

- Common Requirements All Platforms
  If dilution series required in RT-PCR, then include it in array testing.
- Expense
  Dilution series are expensive.
  Useful to have quick screen to reduce the set of candidate standards.
- Quality Checks
  Frequently confirm the quality of all standards.
  Use Agilent BioAnalyzer before and after transport of pools.
- Complexity
  Test standards in complex RNA background.
  Spike pools of standards into human universal reference RNA.
- Platform Differences
  Include reverse-fluor hybridizations in two-color arrays.

Testing Design



Phases of Testing

- Phase I – Replicates of Two RNA Pools

  Quick Screen for:

  - Reproducibility
  - Accuracy

- Phase II – Series of Staggered Dilution Pools

  Thorough Review for:

  - Sensitivity
  - Specificity
  - Accuracy

- Phase III – Proposed Final Pools

Microarray Design

- Novel probes/probe sets complementary to candidate standards
  - Restrict to oligonucleotide arrays, not cDNA
  - Useful to test multiple probes/probe sets per transcript
  - Include replicate spots of same probe
  - Represent 5' and 3' regions of the transcript
- Established probes/probe sets complementary to genes expressed in the complex RNA background

Ideal Array = human catalog array modified to contain multiple probes/probe sets for each candidate standard

RT-PCR Primer Design

- Amplicon lengths between 100-200 bp
  Enables same primer sets can be used in SYBR green or target-based detection systems
- Two sets of primers per standard
  Represent both the 5'and 3' regions of standard
- Primers span intron/exon junctions, when possible
  Avoid amplifying genomic DNA
- Optimized for concentration and ratio
  Identifies fluorescent signal from primer dimers or non-specific amplicons

Phases of Testing

- Phase I – Replicates of Two RNA Pools

Candidate	Pool A	Pool B
Tx 01	[High]	[Low]
Tx 02	[Low]	[High]
Tx 03	[High]	[Low]
Tx 04	[Low]	[High]

Quick Screen for:

- Reproducibility
- Accuracy

All candidate transcripts at:

- ***Same two concentrations (Not a test of dynamic range)***
- ***Same relative abundance between pools.***

Both pool A and pool B have same quantity of RNA.

Phase I Testing

- One-Color Arrays
  Spike pools into human universal reference RNA.
  Hybridize four replicates of Pool A.
  Hybridize four replicates of Pool B.
- Two-Color Arrays
  Spike pools into human universal reference RNA.
  Hybridize two replicates with Cy5-Pool A and Cy3-Pool B.
  Hybridize two replicates with Cy3-Pool A and Cy5-Pool B.
- RT-PCR
  Select “normalizer” transcript & determine its copy number.
  Design and optimize two primer sets per transcript.
  Spike pools into human universal reference RNA.
  Run duplicate RT-PCR reactions for each primer set.
  Determine copy number for transcript by comparing to “normalizer”.

Four Measures per Pool. Compare Pool A / Pool B.

Phases of Testing

- Phase I – Replicates of Two RNA Pools

Candidate	Pool A	Pool B
Tx 01	[High]	[Low]
Tx 02	[Low]	[High]
Tx 03	[High]	[Low]
Tx 04	[Low]	[High]

Quick Screen for:

- Reproducibility
- Accuracy

- Phase II – Series of Staggered Dilution Pools

Candidate	Pool A	Pool B	Pool C	Pool D
Tx 01	0	0.1	1	10
Tx 02	0.1	1	10	0
Tx 03	1	10	0	0.1
Tx 04	10	0	0.1	1

Thorough Review for:

- Sensitivity
- Specificity
- Accuracy

Transcript concentrations 0.1 – 1,000 copies per cell

Expression ratios 0.01 to 100 (if resources permit)

Phases of Testing

- Phase I – Replicates of Two RNA Pools

Candidate	Pool A	Pool B
Tx 01	[High]	[Low]
Tx 02	[Low]	[High]
Tx 03	[High]	[Low]
Tx 04	[Low]	[High]

Quick Screen for:

- Reproducibility
- Accuracy

- Phase II – Series of Dilutions (Latin Squares)

Candidate	Pool A	Pool B	Pool C	Pool D
Tx 01	0	0.1	1	10
Tx 02	0.1	1	10	0
Tx 03	1	10	0	0.1
Tx 04	10	0	0.1	1

Thorough Review for:

- Sensitivity
- Specificity
- Accuracy

- Phase III – Proposed Final Pools

Confirm performance in multiple labs under a variety of conditions.

Outstanding Issues

- BioAnalyzer may not be sufficient for pool QC.
  - Presence of yeast tRNA carrier
  - Pool of 21 Class A transcripts 700-800 nt
- No distinction between Class A vs. Class B or purified vs. encapsulated standards.
- Inconsistent with 4 pools defined for kit 2.
  - Pool 1 =21 Class A Pool 2 = 21 Class A
  - Pool 3 = 21 Class B Pool 4 = 21 Class B
- Need agreement with Acceptance Criteria.
  - Test sensitivity from 0-10,000 copies and ratios from 1:1 to 1:50
  - Define as “Molecules mRNA/100,000” or “parts per million”
- Analysis methods not defined.
  - Reproducibility = Correlation Coefficient at probe level or Percent Signal Change at probe set level