Issues Concerning Extraction Efficiency, Methods, and Direct dPCR

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April 10, 2013
Sample Prep & Target Enrichment
Boston, MA
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Agenda

• Extraction
  – Efficiency (relative vs. absolute)
  – Overview of traditional methods

• Alternates to traditional extractions
  – Liquid based
  – Direct PCR methods

• Direct digital PCR
  – NIST experiences
  – Considerations
Extraction Efficiency

- Relative: compared to another technique
  - New technique > Organic extraction

Analyze for best option:
- Efficiency
- Consistency
Extraction Efficiency

• Absolute: compared to amount of input material

• Mumy et al found ~ 15 % efficiency using 3 commercial kits (range 0 % to 45 %)
  – Lambda DNA in plasmid

New Extraction Method

Sample + known amount of control DNA

Detection

Analyze method:
• Efficiency
• Consistency
DNA Extractions

• Steps:
  – Lysis
  – Separation
  – Purification/wash
  – Recovery

• Benefits
  – Clean DNA

• Limitations
  – No method is 100 % efficient

https://www.beckmancoulter.com/
DNA Extractions

• Steps:
  – Lysis
  – Separation
  – Purification/wash
  – Recovery

 90 % efficient

• Benefits
  – Clean DNA

• Limitations
  – No method is 100 % efficient

Efficiency probably not uniform. But we have to consider that lysis may not be 100 % efficient.

Overall 66 % efficient

https://www.beckmancoulter.com/
Alternate Methods

• Liquid based methods
  – E.g. DNAzol Direct
    • Add reagent to sample
    • Incubate
    • Add directly to PCR

• Benefits
  – All DNA contained in one tube

• Limitations
  – Reagents may not lyse all cell or virus particles
  – Regents may contain PCR inhibitors
    • 1/10 dilution required

http://www.mrcgene.com/dnazoldirect.htm
Extraction Efficiency

• People
  – Training
  – Education
  – Motivation
  – Sleep

• Robots
  – Set-up
  – Maintenance

Things companies don’t tell you.

Direct PCR

- Sample added directly to PCR mix
- Hot start used as lysis method
- Polymerases resistant to inhibition

- Thermo Scientific – Phusion polymerase
  - “Tolerant of many PCR inhibitors”
  - End point PCR protocols
  - Research Use Only
  - No 5’ to 3’ nuclease activity (not suitable for TaqMan probes)
1) Create a PCR mastermix as if for qPCR

2) Aliquot across 100s or 1000s of wells

3) Thermal cycle as if for qPCR & count wells with detectible amplification at any cycle

4) Use Poisson statistics to determine concentration of starting material
Direct dPCR

1) Create a PCR mastermix as if for qPCR

Virus particles instead of template DNA

2) Aliquot across 100s or 1000s of wells

3) Thermal cycle as if for qPCR & count wells with detectible amplification at any cycle

Hot start to lyse virus particles

Exhaustive cycling to ensure lysis & amplification of all target molecules

4) Use Poisson statistics to determine concentration of starting material

pg/μL
1st experiment direct dPCR

- NIST standard protocol Fluidigm 12.765
  - 10 minute hot start and 60 cycles
- Many late amplifications – inefficient lysis

0.03% of amplifications were in the last 10 cycles
1 out of 3763

3.6% of amplifications were in the last 10 cycles
126 out of 3476
1st experiment direct dPCR

- Are we detecting all virus particles?
  - No, late amplifications indicate the hot start is inefficient at lysing viruses

- Solution add more cycles & lysing steps
  - 10 min hot start
  - Every 5 cycles 2 min at 95 °C (first 25 cycles)
  - 85 cycles total
2nd series direct dPCR

- Extra incubations at 95 °C
- 85 cycles total

Extracted DNA

- 0.04% of amplifications were in the last 10 cycles
- 1 out of 2443

Virus particles

- 0.75% of amplifications were in the last 10 cycles
- 28 out of 3739
2\textsuperscript{nd} series direct dPCR

- Still some evidence that all virus particles have not been lysed
  - Additional cycles may not be enough
- Solution: run excessive number of cycles
  - 100 cycles with extra “hot starts”
- Question: will enzyme (Taq Gold – ABI Gene Expression MM) be active at 100 cycles?
Stress Test Polymerase

- Master mix (sans DNA) cycled on standard thermal cycler
- Template DNA added
- Run qPCR on 7500

Conclusion: cycling has little to no effect on polymerase activity. Plateau likely due to consuming dNTPs.

Likely edge effects

NTCs

N=1
100 cycle direct dPCR

0.04 % of amplifications were in the last 10 cycles
1 out of 2446

0.20 % of amplifications were in the last 10 cycles
7 out of 3501
Concentration

• Do additional cycles change result?

![Graph showing concentration over number of PCR cycles](image-url)
Considerations

• Adding cycles adds time & reduces throughput

• Exhaustive cycles gives confidence that all DNA molecules present were amplified

• Principle of diminishing returns
  – Rare very late amplifications may not be significant

• End point systems: Are additional cycles significantly changing the measured concentration?
Considerations (cont.)

• What are you trying to do?
  – Quantifying standard – correct answer
  – Patient sample – would change/variation affect medical decisions?
    • Is ±0.5 log close enough? ±5%? ±1%?

• Dead volume – portion of the sample is not analyzed

<table>
<thead>
<tr>
<th></th>
<th>Fluidigm 12.765</th>
<th>Bio-Rad QX100</th>
<th>Life Technologies Quant Studio 3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input volume</td>
<td>8 µL</td>
<td>20 µL</td>
<td>variable</td>
</tr>
<tr>
<td>Volume analyzed</td>
<td>4.59 µL</td>
<td>10 to 18 µL</td>
<td>up to 20 µL</td>
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<tr>
<td>% Analyzed</td>
<td>57%</td>
<td>50 to 90 %</td>
<td>up to 100 %</td>
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Future Directions

• Correlate particle (or cell) count with direct dPCR measurement
• Estimate of absolute extraction efficiency comparing direct dPCR with extraction followed by dPCR.
Conclusions

• Direct dPCR may be acceptable with heat lysis

• Modifications may be necessary
  – Additional cycles
  – Additional heating (lysing) steps
  – Polymerases resistant to inhibition with 5’ to 3’ nuclease activity

• Purpose and required accuracy may affect optimization scheme
Questions

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