**Plasmid extraction from human fecal samples**

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| Methods to investigate plasmidome | Concept | Pros | Considerations |
| Transposon-aided capture of plasmids | Digest chromosomal DNA, insert transposon w/ selectable marker into plasmid & transform into bacteria for extraction. | -Captures plasmids without selectable marker or those that cannot replicate | -Requires *E. coli* to maintain plasmid after transformation  -Excludes linear and larger plasmids  -Low throughput |
| Multiple displacement amplification | Digest chromosomal DNA, amplify circular plasmids with phi29 polymerase | -Unspecific amplification allows identification of even minority plasmids | -Excludes linear and larger plasmids, though latter may be mitigated via electroelution step (refer to ref 3)  -usually Yields v. pure (high 90s, allow for discovery of new small plasmids) |
| Exogenous extraction (biparental culture) | Isolate and generate donor culture from bacteria, mix with recipient culture; select transconjugants and extract plasmid | -Most consistent method in comparison studies  -Should extract wide range (e.g., integrons, large plasmids)  -Can extract linear plasmids | -Only collect plasmids from culturable bacteria  -Selects for plasmids with high conjugative abilities and can stably replicate |
| In-house transformation & extraction | Extract DNA, transform into bacteria through electroporation (w/o transposon), culture and extract plasmid | -Avoid biases from culture methods and amplification | -Uncertain if electroporation/transformation method only will allow efficient transfer of plasmid into recipient |
| Additional:  Long Read Sequencing (untreated sample) | Extract DNA and take for long-read sequencing, select samples based on interesting samples found during functional metagenomic screens or samples w/ promising quality of DNA | -Avoids biases from any current plasmid extraction method  -Should accurately cover sequences even for larger proteins | -Old samples may be too degraded for LRS to be utilized  -Functional metagenomic will not cover full samples, may need other criteria to select samples for LRS  -Harder to do downstream experiments with, will need to extract separately |

Protocols for plasmid extraction in brief

1. TRACA plasmid extractionn
2. Remove chromosomal DNA with plasmid-safe DNase
3. Amplify genes (PCR)
4. Insert transposon w/ selectable marker into plasmid
5. Transform bacteria, select via transposon marker
6. Extract plasmid
7. Multiple displacement amplification
8. Extract DNA from sample
9. Run plasmid DNA along gel and conduct electroelution
10. Remove sheared DNA with plasmid-safe DNase for desired plasmid sizes
11. Amplification of varied sizes of plasmids w/ Phi29 polymerase
12. Exogenous extraction (biparental culture)
13. Pool fecal samples into broth and incubate overnight
14. Centrifuge pellet and resuspend to establish donor culture
15. Set up selective markers for recipient cultures (e.g., select w/ antibiotics to purify cultures)
16. Mix donor and recipient cultures, pellet; resuspend and apply onto filters w/ plate count agar & cycloheximide (prevents fungal growth); also do with donor culture and recipient to establish background controls
17. Incubate plate, retrieve filters and vortex to detach cells
18. Select cells for transconjugant
19. Amplify/culture for plasmid isolation/sequencing
20. Transformation & extraction (in-house protocol)

Relevant readings

1. <https://www-ncbi-nlm-nih-gov.eproxy.lib.hku.hk/pmc/articles/PMC6100392/#B14>
   1. Comparison between plasmid extraction methods for broiler cecal samples, exogenous extraction most consistent; multiple displacement amplification captured broadest range of plasmids but inconsistent yield between samples.
2. <https://academic.oup.com/femsec/article/92/6/fiw075/2470068?login=true>
   1. Protocol for exogenous extraction (bi-parental culture)
3. <https://pubmed-ncbi-nlm-nih-gov.eproxy.lib.hku.hk/25116381/>
   1. Electroelution step during MDA to improve yield for large plasmids
4. <https://www-ncbi-nlm-nih-gov.eproxy.lib.hku.hk/pmc/articles/PMC4302988/>
   1. Review of current methods to extract plasmids from complex samples
      1. Proposes that most efficient method is to use long read sequencing on promising, untreated samples to retrieve whole genome & mobilome
5. <https://www-ncbi-nlm-nih-gov.eproxy.lib.hku.hk/pmc/articles/PMC7678069/>
   1. MGE annotation from metagenomic data after collecting swine samples
   2. Also uses WAFFLE to predict HGT events and which events involve resistance genes
6. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8182900/>
   1. Dutch traveler cohort
   2. Annotation of contigs against MGEs conducted
7. <https://pubmed-ncbi-nlm-nih-gov.eproxy.lib.hku.hk/26507767/>
   1. Annotation of int1 as proxy for presence of integrons, also demonstrates high abundance of MGEs even in infant microbiota
8. <https://www.nature.com/articles/s41396-019-0446-4>
   1. Hi-C sequencing was used to identify which MGEs were associated with which bacteria, identifying which species were major reservoirs of MGEs during travel.
9. <https://www-ncbi-nlm-nih-gov.eproxy.lib.hku.hk/pmc/articles/PMC7476777/>
   1. Review overview of plasmid extraction methods
10. <https://www-ncbi-nlm-nih-gov.eproxy.lib.hku.hk/pmc/articles/PMC6907783/>
    1. Bioinformatic program shows that lots of MGEs in gut samples may help with niche environmental adaptations
    2. HGT usually within phyla
11. <https://www.frontiersin.org/articles/10.3389/fmicb.2014.00765/full>
    1. Mating protocol that inspired ref 2

Hypotheses:

1. Traveling leads to prominent changes in meta mobilome, which, in turn, should lead to changes in the community’s resistome
2. The gain/loss of these MGE-associated resistance genes correlate with the environment during travel, including diet, activities/events, antibiotic usage in the area and personal hygiene
3. Several MGEs (ideally novel) are prominent carriers of resistance genes, and their presence can be tied:
   1. to other traveler cohorts, demonstrating the gain of resistance genes is driven by travel
   2. to other resident/citizen cohorts, demonstrating that gain of MGEs is destination specific
4. Wet lab experiments should also demonstrate the ease of transmission of these MGEs and transmission of these resistance genes
   1. (For the far future) Ideally, mice experiments should also be conducted to demonstrate dissemination of these MGEs, and more importantly, how intake of antibiotics might affect communities differently now that resistance genes are acquired

Downstream workflow:

1. After metagenomic sequencing, use annotation techniques identified in ref 5,6 to annotate and identify samples with resistance-containing MGEs
2. Extract MGEs from same samples and screen
   1. Ideally do bi-parental culture to verify conjugative abilities, but may select only for plasmids that can be maintained in recipient culture, and from hosts that can be effectively cultured
      1. Can circumvent issues with recipient culture by using a mock community culture rather than pure *E. coli*
3. Take samples identified from (1) for LRS
   1. If low no. Of samples (due to most having low quality etc.), then sequence each sample individually (max~ 10-20)
   2. If high no. Of samples available, then pool in following priorities until appropriate no. Reached
      1. By timepoint
      2. By Region (destination visited)
      3. By time collected
      4. By diet
   3. Ideally also sequence recipient cultures from (2) to verify if any non-plasmid elements have been successfully transferred (though screening might be enough)
      1. To see which MGEs are responsible for conferring different traits
4. Compare extracted MGEs with other traveler and residential cohorts (according to travel destination) to investigate prominence of these MGEs and their role in driving resistome dissemination.

Summary from meeting – functional metagenomics:

1. Pool samples from countries rather than regions
2. Fragment and select based on sizes
3. Transform into competent cells
4. Screen with antibiotic plates
5. Take clones from each plate and grow into adequate amounts
6. Extract DNA, amplify based on vectors & do long read sequencing

Plasmidome:

1. Annotate particular resistance genes and nearby MGEs
2. Identify isolates of samples with those MGEs and screen functionally to identify role