Off-Target Analysis：
Identification, Verification and Compliant Testing

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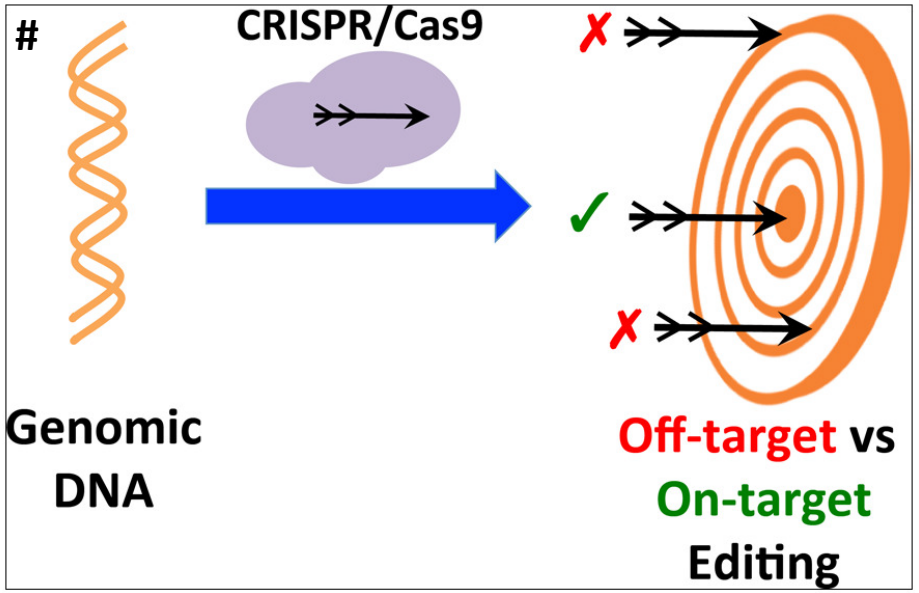
Off-Target Editing

Source of Off-Target Editing:

- ✓ Sequence homology of gRNA
- ✓ Specificity and purity of nuclease
- ✓ Synthesis error of gRNA
- ✓ Residual gRNA from previous synthesis
- ✓ Cell type specific (e.g. chromosome accessibility)



https://pubs.acs.org/cms/10.1021/acssynbio.7b00270/asset/images/acssynbio.7b00270.social.jpeg_v03

Off-Target Editing Identification

	In Vivo (in cells)	*In Vitro* (cell-free)	In Silico Prediction
Purity and specificity of nuclease	Yes	Yes	No
Sequence homology of gRNA	Yes	Yes	Yes
Synthesis error of gRNA	Yes	Yes	No
Synthesis purity of gRNA	Yes	Yes	No
Cell type specific	Yes	No	No
Signal/Noise Ratio	High	Low	N/A
Limitation	dsODN Incorporation	High Noise	Sequence Dependent
Representative Method	GUIDE-Seq	SITE-Seq	Many

GGC Recommendation: G-GUIDE (with UMI) + In Silico (2+ algorithm)

Off-Target Verification

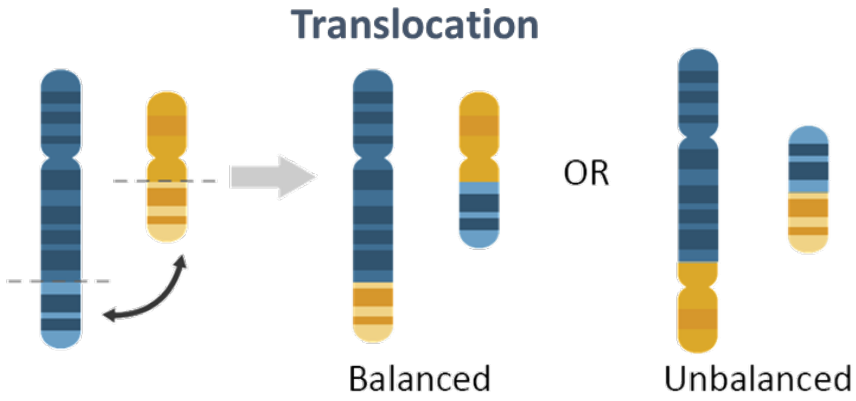
	Targeted - Amplicon	Targeted - Probe	WGS
Target Coverage	High	High	Low
LOD/LOQ	High	Mid	Low
Accuracy	High	Mid	Low
Precision	High	High	Mid
Genomic Region Coverage	Low	Medium	High
Turnaround Time	Fast	Mid	Slow
Cost	Low	Mid/Low	High

GGC Recommendation: Targeted Amplicon with UMI

Off-Target - Translocation

Genomic Translocation:

- ✓ Frequency and Functional Impact
- ✓ Increasing requirement in IND
- ✓ Requires high detection sensitivity:
  - ❖ on-on : 0.1% ~ 0.5 %
  - ❖ on-off : 0.001% ~ 0.03%



Challenges in Off-Target Identification and Verification

- ✓ How sensitive is sensitive enough? 0.1% vs. 0.01%

  Insertion, Deletion vs. Substitution.

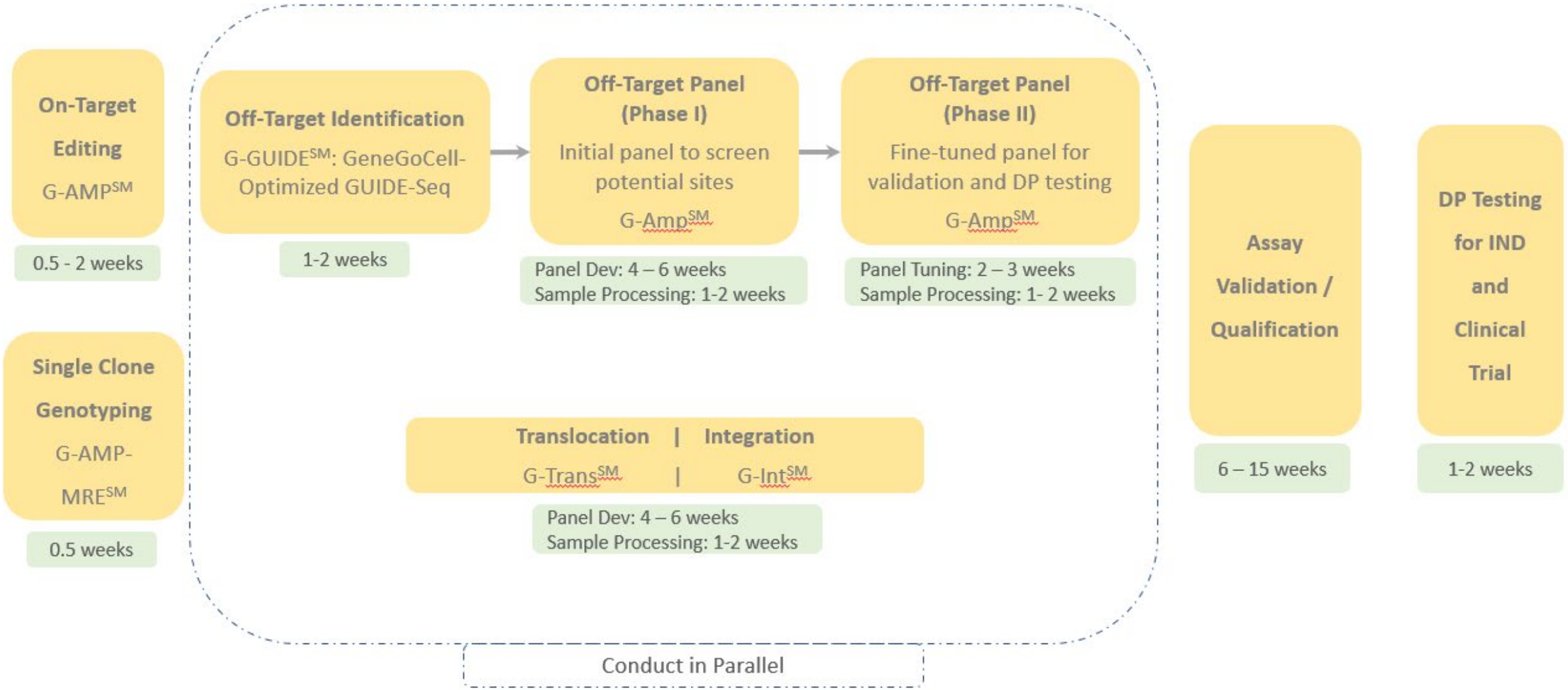
- ✓ Complex vs. Clonal
- ✓ Sequencing depth vs. effective depth – importance of UMI
- ✓ Ethnicity background – private SNPs.
- ✓ PacBio HiFi read vs. Illumina short read

Compliant Testing

Test Qualification/Validation:

- ✓ Linearity
- ✓ LOD/LLOD and LOQ/LLOQ
- ✓ Accuracy: sensitivity and specificity
- ✓ Precision: intra-run repeatability and inter-run reproducibility
- ✓ Robustness (optional)

R&D → IND → Clinical Trial



Thank you!