PATHOGENIC CRITERIA		
Criteria	**Criteria Description**	**Specification**
VERY STRONG CRITERIA		
PVS1	Null variant in a gene where loss of function is a known mechanism of disease.	None
PM3_Very Strong	For recessive disorders, detected in *trans* with a pathogenic variant. • 4 compound heterozygotes with 3 P/LP variants OR • 2 compound heterozygotes with 2 P/LP variants AND 4 homozygotes OR • 3 compound heterozygotes with 2 P/LP variants AND 2 homozygotes	Strength
STRONG CRITERIA		
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.	None
PS2	*De novo* (paternity confirmed) in a patient with the disease and no family history.	None
PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect • PAH enzyme activity assay demonstrating enzyme activity <50% • RT-PCR evidence of missplicing for non-canonical intronic variants	Disease-Specific
PS4	*The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls.*	*N/A*
PM3_Strong	For recessive disorders, detected in *trans* with a pathogenic variant. • Compound heterozygous with 2 P/LP variants OR • Compound heterozygous with 1 P/LP variant AND 2 homozygotes	Strength
PP1_Strong	Co-segregation with disease in multiple affected family members:	Strength

	• 3 affected segregations + 0 unaffected segregations OR • 2 affected segregations + 3 unaffected segregations	
MODERATE CRITERIA		
PM1	*Located in a mutational hot spot and/or critical and well-established functional domain.*	*N/A*
PM2	Absent/rare from controls in an ethnically-matched cohort population sample. • Threshold: <0.0002 (0.02%).	Disease-Specific
PM3	For recessive disorders, detected in *trans* with a pathogenic variant.	None
PM4	Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants.	None
PM5	Missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before.	None
PM6	*Confirmed de novo without confirmation of paternity and maternity.*	*N/A*
PP4_Moderate	Plasma Phe >120 µmol/L and exclusion of a defect of BH4 cofactor metabolism.	Strength; Disease-Specific
PP1_Moderate	Co-segregation with disease in multiple affected family members • 2 affected segregations + 0 unaffected segregations	Strength
SUPPORTING CRITERIA		
PP1	Co-segregation with disease in multiple affected family members • 1 affected family member + 3 unaffected segregations	Disease-Specific
PP2	*Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease.*	*N/A*
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product	None
PP4	Phenotype specific for disease with single genetic etiology.	None

PP5	*Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation*	*N/A*
PM3_ Supporting	Detected *in trans* with another variant: • 2 compound heterozygotes (with VUS *in trans*) • 2 homozygotes (allele drop out excluded)	Strength

BENIGN CRITERIA

Criteria	Criteria Description	Specification
STAND ALONE CRITERIA		
BA1	Allele frequency above 0.015 (1.5%)	Disease-Specific
STRONG CRITERIA		
BS1	Allele frequency greater than expected for disease (>0.002, 0.2%)	Disease-Specific
BS2	Observed in the homozygous state in a healthy adult	None
BS3	*Well-established in vitro or in vivo functional studies shows no damaging effect on protein function*	*N/A*
BS4	Lack of segregation in affected members of a family.	None
SUPPORTING CRITERIA		
BP1	*Missense variant in gene where only LOF causes disease*	*N/A*
BP2	*Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder; or observed in cis with a pathogenic variant in any inheritance pattern.*	*N/A*
BP3	*In-frame deletions/insertions in a repetitive region without a known function*	*N/A*
BP4	Multiple lines of computational evidence suggest no impact on gene or gene product	None
BP5	Variant found in a case with an alternate molecular basis for disease	None
BP6	*Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation*	*N/A*
BP7	A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved.	None
BS3_Supporting	Well-established *in vitro* or *in vivo* functional studies shows no damaging effect on protein function • Enzyme activity >85%	Strength; Disease-Specific

Key: **Disease-Specific:** Disease-specific modifications based on what is known about PAH; **Strength:** Increasing or decreasing strength of criteria based on the amount of evidence; **N/A:** not applicable for PAH; **None:** no changes made to existing criteria definitions.

VERY STRONG EVIDENCE OF PATHOGENICITY

PVS1

Null variant (nonsense, frameshift, canonical +/-1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where loss of function (LOF) is a known mechanism of disease

Caveats:

- Use caution interpreting LOF variants at the extreme 3' end of a gene
- Use caution with splice variants that are predicted to lead to exon skipping but leave the remainder of the protein intact

PAH: PVS1 is applicable as described

PM3_VeryStrong

Detected in *trans* with a pathogenic variant

PAH:

- 4 Compound heterozygotes with 3 different pathogenic/likely pathogenic variants
- 2 Compound heterozygotes with 2 different pathogenic/likely pathogenic variant & 4 homozygotes
- 3 Compound heterozygotes with 2 different pathogenic/likely pathogenic variant & 2 homozygotes

STRONG EVIDENCE OF PATHOGENICITY

PS1

Same amino acid change as a previously established pathogenic variant regardless of nucleotide change

PAH: PS1 is applicable as described

PS2

De novo (both maternity and paternity confirmed) in a patient with the disease and no family history

Note: Confirmation of paternity only is insufficient. Egg donation, surrogate motherhood, errors in embryo transfer, *etc.* can contribute to non-maternity

PAH: Only applicable when proband has a known pathogenic variant in trans with the *de novo* variant

PS3 Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product

PAH:

- *In vitro* enzyme activity <50% compared to wild type control.
  - Expression systems placing the mutant (and wild-type) cDNAs into plasmid vectors and introducing these into human or other mammalian host cells, which is the closest available approximation to the *in vivo* situation. (e.g. COS cells)(Trunzo, et al. Gene. 2016 594:138-143.PMID: 27620137).
- RT-PCR evidence of missplicing
  - For non-canonical splicing variants, RT-PCR evidence demonstrating transcripts of alternative length or specific intron or exon inclusion/exclusion. These studies can be performed in patient derived cells, or by placing the mutant genomic DNA into plasmid vectors and introducing these into human or other mammalian host cells. Assays should demonstrate defective splicing with RT-PCR analysis or RNA sequencing to confirm alternative transcripts.

PS4 The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls

PAH: This criterion is not applicable for PAH. For proband counting, use PM3 criterion

PM3_Strong Detected in *trans* with a pathogenic variant

PAH: Use SVI thresholds

- Compound heterozygous with 2 different pathogenic/likely pathogenic variants
- Compound heterozygous with pathogenic/likely pathogenic variant & 2 homozygotes

PP1_Strong Co-segregation with disease in multiple affected family members

PAH:

- 3 affected segregations + 0 unaffected segregations
- 2 affected segregations + 3 unaffected segregations

MODERATE EVIDENCE OF PATHOGENICITY

PM1 Located in a mutational hot spot and/or critical and well-established functional domain (*e.g.* active site of an enzyme) without benign variation
PAH: Not applicable

PM2 Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes or ExAC
PAH threshold: <0.0002 (0.02%). The 0.0002 cutoff is based on disease frequency of 1:12,000 and the most common PAH pathogenic variant, R408W, the ExAC frequency is 0.0006594 (ExAC MAF: 0.001109 74/66718 European Non-Finnish) and gnomAD overall: 0.0009056 (gnomAD MAF: 0.001728 219/126,700 European Non-Finnish)

PM3 For recessive disorders, detected in *trans* with a pathogenic variant
Note: This requires testing of parents (or offspring) to determine phase
PAH: PM3 is applicable as described

PM4 Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants
PAH: PM4 is applicable as described

PM5 Missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before
PAH: PM5 is applicable as described

PM6 Assumed *de novo*, but without confirmation of paternity and maternity
PAH: Not applicable

PP4_Moderate Patient's phenotype or family history is highly specific for a disease with a single genetic etiology
PAH:

- A plasma phenylalanine concentration persistently above 120µmol/L (2mg/dL), and either normal urine pterins and normal DHPR activity, or sequencing of genes in the BH4 cofactor metabolism pathway to exclude a defect of BH4 cofactor metabolism.

PP1_Moderate Co-segregation with disease in multiple affected family members

PAH:

- 2 affected segregations + 0 unaffected segregations

SUPPORTING EVIDENCE OF PATHOGENICITY

PP1 Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease

PAH:

- 1 affected segregation + 3 unaffected segregations

PP2 Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease

PAH: Not applicable

PP3 Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc)

PAH: PP3 is applicable as described

PP4 Patient's phenotype or family history is highly specific for a disease with a single genetic etiology.

PAH:

- A plasma phenylalanine concentration persistently above 120µmol/L (2mg/dL) without analysis of urine pterins, DHPR activity, or sequencing to exclude defects of BH4 cofactor metabolism.

PP5 Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation

PAH: Not applicable

PM3_Supporting Detected in *trans* with a pathogenic variant

PAH: Use SVI thresholds

- 2 compound heterozygotes (with a VUS *in trans*)
- 2 homozygotes (with allele drop out excluded)

STAND ALONE EVIDENCE OF BENIGN IMPACT

BA1 Allele frequency is above 5% in Exome Sequencing Project, 1000 Genomes, or ExAC

PAH: An allele frequency ≥0.015 (1.5%), which is calculated with genetic heterogeneity of 90% to account for defects of BH4 metabolism, and penetrance of 80% to account for individuals who come to attention after becoming clinically symptomatic.

STRONG EVIDENCE OF BENIGN IMPACT

BS1 Allele frequency is greater than expected for disorder
PAH: An allele frequency ≥0.002 (0.2%)

BS2 Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder with full penetrance expected at an early age.
PAH: Only to be used when variant is observed in the homozygous state in a healthy adult

BS3 Well-established *in vitro* or *in vivo* functional studies show no damaging effect on protein function or splicing.
PAH: See BS3_Supporting

BS4 Lack of segregation in affected members of a family
PAH: BS4 applicable as described

SUPPORTING EVIDENCE FOR BENIGN IMPACT

BP1 Missense variant in a gene for which primarily truncating variants are known to cause disease
PAH: Not applicable

BP2 Observed in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder; or observed in *cis* with a pathogenic variant in any inheritance pattern
PAH: Not applicable

BP3 In-frame deletions/insertions in a repetitive region without a known function
PAH: Not applicable

BP4 Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.)
PAH: BP4 applicable as described

BP5 Variant found in a case with an alternate molecular basis for disease
PAH: BP5 applicable as described

BP6 Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation
PAH: Not applicable

BP7 A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved
PAH: BP7 applicable as described

BS3_Supporting Well-established *in vitro* or *in vivo* functional studies show no damaging effect on protein function or splicing.
PAH:

- *In vitro* enzyme activity >85% compared to wild type.
    - Expression systems: placing the mutant (and wildtype) cDNA into plasmid vectors and introducing these into host cells. Transiently transfected human or other mammalian host cells are the closest available approximation to the *in vivo* situation. (e.g. COS cells) (Trunzo, et al. Gene. 2016 594:138-143)

RULES FOR COMBINING PATHOGENIC CRITERIA

Pathogenic

1. 1 Very Strong (PVS1, PM3_VeryStrong) AND
    a. ≥1 Strong (PS1-PS4, PM3_Strong, PP1_Strong) OR
    b. ≥2 Moderate (PM1-PM6, PP4_Moderate, PP1_Moderate) OR
    c. 1 Moderate (PM1-PM6, PP4_Moderate, PP1_Moderate) and 1 Supporting (PP1-PP5, PM3_Supporting) OR
    d. ≥2 Supporting (PP1-PP5, PM3_Supporting)
2. ≥2 Strong (PS1-PS4, PM3_Strong, PP1_Strong) OR
3. 1 Strong (PS1-PS4, PM3_Strong, PP1_Strong) AND
    a. ≥3 Moderate (PM1-PM6, PP4_Moderate, PP1_Moderate) OR
    b. 2 Moderate (PM1-PM6, PP4_Moderate, PP1_Moderate) AND ≥2 Supporting (PP1-PP5, PM3_Supporting) OR
    c. 1 Moderate (PM1-PM6, PP4_Moderate, PP1_Moderate) AND ≥4 Supporting (PP1-PP5, PM3_Supporting)

Likely Pathogenic

1. 1 Very Strong (PVS1, PM3_VeryStrong) AND 1 Moderate (PP1-PP5, PM3_Supporting) OR
2. 1 Strong (PS1-PS4, PM3_Strong, PP1_Strong) AND 1-2 Moderate (PM1-PM6, PP4_Moderate, PP1_Moderate) OR
3. 1 Strong (PS1-PS4, PM3_Strong, PP1_Strong) AND ≥2 Supporting (PP1-PP5, PM3_Supporting) OR
4. ≥3 Moderate (PM1-PM6, PP4_Moderate, PP1_Moderate) OR
5. 2 Moderate (PM1-PM6, PP4_Moderate, PP1_Moderate) AND ≥2 Supporting (PP1-PP5, PM3_Supporting) OR
6. 1 Moderate (PM1-PM6, PP4_Moderate, PP1_Moderate) AND ≥4 Supporting (PP1-PP5, PM3_Supporting)

RULES FOR COMBINING BENIGN CRITERIA Benign

1. 1 Stand-Alone (BA1) OR
2. ≥2 Strong (BS1-BS4)

Likely Benign

1. 1 Strong (BS1-BS4) and 1 Supporting (BP1-BP7, BS3_Supporting) OR
2. ≥2 Supporting (BP1–BP7, BS3_Supporting)